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Effects of a moderate and aggressive implant strategy on the rumen microbial community and metabolome in steers

Madison Henniger

University of Tennessee, Knoxville, mhennige@vols.utk.edu

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To the Graduate Council:

I am submitting herewith a thesis written by Madison Henniger entitled "Effects of a moderate and aggressive implant strategy on the rumen microbial community and metabolome in steers." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Phillip R. Myer, Major Professor

We have read this thesis and recommend its acceptance:

Kyle J. McLean, Liesel G. Schneider

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

**EFFECTS OF A MODERATE AND AGGRESSIVE
IMPLANT STRATEGY ON THE RUMEN
MICROBIAL COMMUNITIES AND METABOLOME
IN STEERS**

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Madison Taylor Henniger
August 2020

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ABSTRACT

The effects of growth-promoting implants have been well-defined for their ability to impact growth in beef cattle. Production-relevant microbiomes in the rumen have also been associated with growth traits. However, the role of implant strategies on the rumen microbiome is not understood. The objective was to determine if varying doses of implant hormones cause gain-associated ruminal microbial community shifts. To assess this, a completely randomized design was used and 336 fall-born steers between 450-470 days of age from the germplasm evaluation population at the US Meat Animal Research Center (Clay Center, NE) were divided into two treatment groups: 1) a moderate implant strategy of Revalor-IS (80 mg trenbolone acetate and 16 mg estradiol) followed by Revalor-S (120 mg trenbolone acetate and 24 mg estradiol) and 2) an aggressive implant strategy of Revalor-IS followed by Revalor-200 (200 mg trenbolone acetate and 20 mg estradiol). Steers were fed the same diet (57.0% dry-rolled corn, 30% wet distiller's grains with solubles, 8.0% alfalfa hay, 4.25% supplement, and 0.75% urea, on a DM basis). Body weights were collected once per month with initial weights of 439.8 ± 43.1 kg. After implants were administered for 84 days, rumen content was collected via orogastric tubing. Samples were sequenced targeting bacterial V1-V3 16S rRNA gene regions, V3-V4 for archaea, and partial 18S rRNA gene of protozoa. Sequences were processed in R utilizing phyloseq with DADA2 and analyzed with DESeq2 to test differential abundances. Untargeted metabolomics was performed on rumen fluid using the UHPLC-HRMS system. Production data between implant strategies was analyzed using a mixed model ANOVA (SASv9.4, Cary, NC) followed by analysis of least square means. Alpha- and beta-diversity between strategies did not differ for bacteria, archaea, or protozoa ($P > 0.05$). Average daily gain was different ($P = 0.01$; 1.72 vs 1.66 ± 0.02 kg, aggressive vs moderate, respectively); however, large microbial community shifts were not associated implant strategy. Two metabolites, acetyllysine and N-acetyloronithine, were significant between implant strategy ($P \leq 0.04$). Understanding associations between the rumen microbiome and implant strategies may allow improvement of growth in beef cattle.

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CHAPTER ONE

LITERATURE REVIEW

Introduction

As of 2017, the world population was approximately 7.5 billion people and growing. By 2100, technology to improve life expectancy and fertility is expected to allow the world population to continue growing, potentially to 8.4 billion people or more (Lutz et al., 2001). Multiple concerns are associated with this growth, such as those of availability of basic necessities, namely food. To provide more food sources to a growing population, beef cattle producers have been assessing varying strategies to increase feed efficiency in their beef herds.

Beef is the largest category of red meat consumed worldwide, due to the high-quality protein, iron and zinc concentration, and presence of B-vitamins (Linseisen et al., 2002). The United States is the world's largest producer of high-quality beef for both domestic and export use (USDA ERS, 2019). In the United States, per capita beef consumption is approximately 26 kilograms [57.3 pounds] annually, with that number rising every year (Drouillard, 2018). Increasingly more important, beef exports have continued to grow through the years. An increase in the world population calls for an increase in sustainably-produced and economically-viable beef.

Approximately 50-70% of input costs in a beef cattle operation go towards feed ingredients (Arthur et al., 2005). In order to reduce production costs, producers target animal feed efficiency to increase the productivity of animals through their conversion of feedstuffs into weight gain. Feed efficiency can be measured and calculated through multiple means, such as average daily gain [ADG], gain-to-feed [G:F] ratios, and residual feed intake [RFI] (Koch et al., 1963). Utilizing feed efficiency information has allowed producers to begin to understand how to maximize input effect and identify any genotypes.

Different management strategies have targeted an increase in overall gain in cattle, such as implanting. These implant strategies overall can improve gain as well as alter feed efficiency while having little effects on carcass quality. Typical market implant strategies contain not only estrogenic compounds but also androgenic compounds for additive growth effects. The hormones present in implants have differing effects on physiological systems, acting directly and indirectly on skeletal muscle and increasing growth factors present in plasma. Overall, management strategies utilizing implants in beef cattle increase producers' revenue by increasing live carcass weights. These implanting technologies are provided by multiple brands, such as Merck Animal Health

[Desoto, KS], with Ralgro and Revalor implants, or Synovex, and consist of different estrogenic and/or androgenic compounds that work together to provide growth within a certain payout period, typically between 170-240 days (Hickman et al., 1994). These implant strategies are not the only factor that improves feed efficiency in beef cattle, as there are different nutritional impacts, genetics, as well as the effects from the rumen microbiome. Implant strategies release hormones over a period of time that can interact with different physiological systems, potentially altering the gut microbiome through interactions with the digestive system.

Commensal microbes present in the rumen microbiome are associated nutrient supply, prevention of disease (Flint et al., 2012), and overall host health (Thomas et al., 2017). In beef cattle, the rumen degrades the majority of feedstuffs consumed through the presence of microbial communities. The presence of bacteria, protozoa, archaea, and fungi in the rumen allow for ruminants to convert feedstuffs into nutrients, providing the primary source of energy to the host. Within the bacterial communities, individual microbes serve the purpose of providing cellulolytic, proteolytic, and lipolytic functionality to break down feedstuffs. The presence of microbial communities in the rumen also provide metabolic activity, primarily through the presence of volatile fatty acids [VFAs], which have been demonstrated to break down feedstuffs into energy for the animal (Cottyn et al., 1968).

More recently, the rumen microbiome has been linked to certain host phenotypes, namely feed efficiency metrics. With the aid of next-generation sequencing technologies, production-relevant taxa are being identified in the gut microbiome and associated with gain in beef cattle, potentially altering host factors to improve feed efficiency (Myer et al., 2015). Productivity of the animal can be heavily impacted by a shift in microbial communities in the rumen, thus demonstrating the microbiome's potential to further influence growth and feed efficiency (Hales et al., 2014, Kim et al., 2014).

While studies have established the impact of growth-promoting implanting strategies on overall weight gain and feed efficiency and separately also how the gut microbiome in cattle plays an important role in host phenotypes and productivity, little information is available to associate the potential relationship between implanting strategies and the rumen microbiome. This manuscript serves to review currently understood effects from implanting strategies in association with impacts from the rumen microbiome to influence weight gain in cattle.

Feed Efficiency in Beef Cattle

Feed efficiency is measured as a gain in body weight that occurs due to conversion of feedstuffs into nutrients over a period of time (Koch et al., 1963). Thus, overall feed efficiency must be computed through multiple factors, which leads to multiple methods of determining feed efficiency. Historically, feed efficiency was measured as a gain-to-feed ratio, in which a larger number was desirable to demonstrate feed efficiency. However, research has aimed towards utilizing RFI as a metric of feed efficiency as there is less possible variation in the calculation (Koch et al., 1963). Separately from feed efficiency metrics, ADG, is a commonly used productivity metric, demonstrating specifically the averaged gain over the course of the study, with a higher gain showing a greater growth. However, ADG does not include feed intake data, and thus cannot be a true predictor of feed efficiency in cattle, but can provide useful data towards calculating other features of feed efficiency.

Importantly, feed efficiency is mediated by multiple factors in ruminants, from genetics (Schenkel et al., 2004, Berry and Crowley, 2013), feed intake and nutrient availability, or environment and management styles. Therefore, there are multiple potential ways to influence feed efficiency in beef cattle. An increase in feed efficiency in cattle would result in lower producer costs and more beef available on the market as a protein source to a growing population, as the change would mean animals ability to convert feedstuffs to gain would improve. Interestingly, beef cattle make an ideal candidate for adjusting growth and meat production due to the multiple factors that affect their feed efficiency, such as the genetic factors, sources of nutrition and additives in the diet, and production management. Genetic selection of beef cattle has occurred over multiple years, primarily to address growth parameters and fertility; genetic improvements in beef cattle that address feed efficiency ideally increase beef cattle production while minimizing quality changes in outputs (Archer et al., 1999).

Differing management strategies can allow for producers to increase feed efficiency in their animals, resulting in increased gain and thus increased revenue. It is important to note that feed efficiency is dependent on multiple factors, such as genetics and nutrition, as well as management. However, implanting animals provides the most return for a producer's management strategy, overall increasing feed efficiency by 5% and daily gains by 10%, allowing this to be the most effective management strategy (Duckett et al., 1996). Without the factors such as a well-formulated diet, implant strategies will not provide the growth expected. These implants can vary in costs, being between \$1 to \$3 in USD, depending on the androgenic and estrogenic compounds present as well as their levels (Mader, 1994).

Implant Technologies and Strategies

Anabolic implants have been approved by the FDA for beef cattle meat production since the 1950s (Bartle et al., 1992). Further, the FDA approved both synthetic hormones for testosterone, estradiol [E₂], and progesterone to be utilized on steers and heifers intended for meat production by 1987 (Preston, 1999). Namely, the approved synthetic estrogenic hormone is zeranol, with other natural forms of estradiol such as estradiol benzoate [EB] commonly used in the implant industry as well. The synthetic androgenic hormones approved are trenbolone acetate [TBA] (Velluz et al., 1967). Notably, the largest increase in growth is when these hormones are applied in a combination of an estrogenic and androgenic compound rather than alone, acting additively. These implant strategies are known to improve ADG by 30% and feed efficiency by 15%, as well as altering carcass qualities such as leanness (Preston, 1999). Multiple studies have found that implanting strategies decrease marbling scores in carcasses (Bartle et al., 1992, Herschler et al., 1995, Platter et al., 2003); however, few studies have found the converse relationship (Gerken et al., 1995, Johnson et al., 1996a). Implant strategies have been classified into low-, medium-, or high-potency to describe their levels of hormones, and contain either coated or non-coated implants to alter the release of hormones. Implants containing more hormones typically cost more money, meaning a producer must make the decision to use a moderate or aggressive implant strategy. Importantly, some implant strategies that contain higher levels of androgenic compounds will increase gain and decrease feed efficiency. Further research is required to understand how androgenic and estrogenic compounds work in low- and high-potency strategies to augment feed efficiency and gain.

Estrogenic Compounds

The first hormonal requirement for implant growth is the presence of estrogenic compounds alone, while an androgenic hormone in combination adds further growth (Preston, 1999). Currently, most implant products have an estrogen base, indicating the requirement of estrogenic activity. Studies have analyzed the effects of just estrogenic activity, finding that non-estrogenic isomers such as *cis*-diethylstilbestrol (Preston et al., 1971, Bradley et al., 1972), and other compounds such as alpha-estradiol, stilbene, estriol, and estrone do not cause the same levels of growth (Preston et al., 1978).

Estradiol is the primary structure of estrogen present in the body and is considered to have strongest estrogenic activity and response. This allows for E₂ to be the main source of estrogenic activity in implants, used in Revalor implant strategies. The other commonly used structure of estrogen is EB, which is considered bioidentical to E₂, acting in similar mechanisms but containing a different benzoate

ester. This compound is utilized in implanting strategies by Synovex, typically combined with progesterone. Similarly to other combinations of growth-promoting hormones, EB/progesterone combinations have been proven to increase feed efficiency and gain in heifers and steers when compared to animals receiving no implant strategies (Mader et al., 1994).

Androgenic Compounds

Androgenic compounds are typically added in combination with an estrogenic compound to have additive growth as well as adjust certain carcass traits. Early research in implant technology demonstrated the effects that sole testosterone played on growth and development of desired carcass qualities (Galbraith and Topps, 1981). Importantly, some implant strategies with higher levels of testosterone have been shown to decrease marbling scores in steers (Smith et al., 1992). When development began of synthetic testosterone, studies found the bioidentical and synthetic versions to be more potent than testosterone alone, making natural testosterone-based strategies low potency and less commonly utilized (Smith and Johnson, 2020). Thus, synthetic analogs of testosterone are more commonly used in implant strategies, such as testosterone propionate and TBA, to address producer desires for growth.

Testosterone propionate was offered as a potential synthetic androgenic compound to use in combination with estrogenic compounds. Synovex implants typically use testosterone propionate in combination with EB for heifers. However, TBA is a commonly used synthetic testosterone due to having 8 to 10 times the anabolic activity and 3 to 5 times the androgenic effect when compared to testosterone propionate (Bouffault and Willemart, 1983). In order to have the most efficient growth utilizing these implant strategies, estrogenic compounds must be added in combination with androgenic compounds to cause additive growth and improved feed efficiency.

Estrogenic and Androgenic Combinations

Previous studies mentioned demonstrate the ability of implant strategies to increase feed efficiency and gain in steers. With the knowledge that the basis of estrogenic activity is required for the additive growth provided by androgenic compounds, these implanting strategies use a combination of both. However, ideal ratios of estrogenic and androgenic combinations still require further research. TBA and E2 ratios have been tested through multiple studies (Bartle et al., 1992, Herschler et al., 1995) in order to find an ideal ratio for growth in cattle. In a study by Herschler et al. in 1992, they noted steers and heifers implanted with a 1:10 ratio of EB/TBA had improved feed efficiency and growth in comparison to a group treated with a 1:5 ratio.

Different combinations with varying levels of estrogenic and androgenic compounds can result in strategies that alter gain by itself or gain and feed efficiency. These strategies, typically considered a moderate or aggressive implant strategy, depend on when the animal is initially implanted and then the level of hormone present in the following implant.

Reimplantation

In order to provide the maximum payout for implant strategies, during the 1980s, long-acting implant strategies were created to allow for extended increases in growth and feed efficiency in steers and heifers (Ferguson et al., 1988, Reuter et al., 2016). These implant strategies aimed to release growth-promoting hormones anywhere between 200-400 days. While these implants proved effective, studies began to examine the decrease in hormone release from implants over time (Lee et al., 1990, Hayden et al., 1992). Multiple studies following the release of these products examined the possibility of reimplantation of the growth-promoting hormones to improve the growth response over a period of time (Bartle et al., 1992, Duckett et al., 1996).

As of recent, cattle intended for meat production are spending more time in feedlots, increasing to greater than 200 days. In order to address this, coated implants with a polymer coating were created to allow for longer payout times, thus delaying release of hormones (Smith et al., 2018). As demonstrated in previously mentioned studies, the hormonal release from implants wanes over time, thus potentially providing an opportunity for a reimplantation strategy. In most feedlot operations, after a 60-120-day period following implantation, cattle will be re-implanted to maintain and improve growth and efficiency responses to the implant strategy. Reimplantation strategies are thought to increase gain response by anywhere between 5-20% depending on the levels of hormones present in the original implant strategy, however not improving feed efficiency response (Preston, 1999). Interestingly, if cattle are re-implanted with a more “aggressive”, or long-lasting and higher dosage, implant strategy following their initial implant, an increased growth response is recorded (Duckett et al., 1996). Thus, reimplantation strategies provide useful increases in growth and feed efficiency to producers when utilized in accordance to specific brand requirements.

Physiological Interactions

In a non-implanted animal, signals for testosterone and estradiol production are given via the pituitary and other endocrine glands. Primarily, the signal for testosterone will spur production of the hormone from the testicles; however, in steers, without testes, testosterone production is severely decreased (Unruh, 1986). Without the increased presence of testosterone, steers will have decreased

growth production in comparison to bulls (Hunt et al., 1991). By proxy, estrogen production is then decreased in steers, as testosterone is converted to estrogen via aromatase (Schulster et al., 2016). The presence of natural testosterone from the testes does not only dictate growth but also behavior and carcass traits. During the finishing phase, bulls exhibit more aggressive behavior which leads to an increase in the prevalence of bruising and carcass shrinkage (Price and Tennesen, 1981). Thus, producers often elect to castrate bulls intended for meat production, altering aforementioned qualities. In order to continue to augment growth in steers, approximately 70% of feedlot cattle producers utilize implant technology (UGA Extension, 2013).

Interestingly, these growth-promoting hormones act on the body in multiple different physiological manners. The presence of androgenic compounds in implants is thought to act directly on muscle receptors, but also indirectly of muscle tissues through other mechanisms. Glucocorticoids have been proven to have a catabolic effect on protein metabolism in muscle tissues, thus indicating that higher circulations of glucocorticoids results in decreased growth in ruminants (Brameld et al., 1998). Implants containing TBA have been shown to decrease serum cortisol and glucocorticoids levels in rats and sheep, as well as cattle, and have established that TBA reduces the adrenocorticotrophic-stimulated release of corticosterone, decreasing adrenal activity (Henricks et al., 1984, Sillence et al., 1987, Jones et al., 1991). With that relationship between androgenic compounds and a decrease in cortisol, higher growth performance has been observed; however, further work is being done to identify the metabolic relationship between plasma cortisol and growth (Purchas et al., 1980).

These androgenic and estrogenic compounds are used in combination for implant strategies in steers, acting indirectly on the pituitary to cause release of bovine growth hormone and directly on skeletal muscle receptors (Preston, 1987). Previous studies have shown that bovine skeletal muscle interacts with these hormones through the presence of free androgen (Snochowski et al., 1981) and estrogen receptors (Meyer and Rapp, 1985), with androgens directly causing growth on receptor sites present in the muscle tissues (Sauerwein and Meyer, 1989). Testosterone has been further studied to identify its role in mechanisms associated with growth hormones [GH], however needs further research to identify specific pathways and interactions. Studies have hypothesized the potential of estrogens acting indirectly on growth through regulating plasma growth hormones, insulin, and thyroid hormones; however, research regarding these relationships can often be conflicting between studies and needs further analysis (Preston, 1975, Johnson et al., 1996b).

These studies focus on analyses of serum to further identify mechanisms by which growth-promoting implant strategies improve gain and feed efficiency in animals through estrogenic activity. Insulin-like growth factor I [IGF-I] is a peptide that

works in signaling for proliferation of multiple cell types, which includes muscle cells, and is highly similar in structure and function to insulin (Florini et al., 1991). Likely, the primary source of IGF-I comes from the liver, however also has been found in large quantities and has multiple binding sites in muscle cells and tissues, leading to assumptions that it plays a role in muscle growth (Froesch and Zapf, 1985). This somatotropin-dependent anabolic peptide is likely increased in circulation through E₂ reportedly increasing the concentrations of somatotropins, which act to regulate IGF-I through different biological activity (Grigsby and Trenkle, 1986, Breier et al., 1988). Interestingly, somatotropins are increased with only estrogenic implants, while combinations with androgenic compounds solely increased IGF-I concentrations (Lee et al., 1990, Hunt et al., 1991, Hangerholt et al., 1992). IGF-I and other related peptides act similarly to insulin, which have been associated with growth related to skeletal muscle (Froesch and Zapf, 1985). Thus, the IGF-I and consequently insulin concentrations in serum are highly likely to be related to growth factors such as gain in cattle.

Summarized, anabolic implants have been identified to increase gain metrics in cattle by increasing protein deposition and decreasing fat once reaching a certain biological weight (Guiroy et al., 2002). Multiple studies have been performed showing the increase in growth as well as increase in feed intake and daily gain from implanting cattle (Reinhardt, 2007). Along with that, they influence multiple serum hormones and metabolites that result in gain in cattle (Johnson et al., 1996b). Implants appear to be more efficient with absorbed energy through feedstuffs, allowing cattle to have a higher finished body weight. This weight can be increased from 14 to 42 kg in steers, translating to more carcass weight that improves producer returns (Guiroy et al., 2002). Receptors for steroid hormones are located throughout multiple physiological systems in cattle and play important roles in many biological responses that are commonly measured through resulting products from hormonal signaling (Filardo and Thomas, 2012). These receptors could indicate potential hormonal activity with different physiological systems that have not been widely identified. While implant-related hormones have been intensively studied, further interactions regarding intracellular signaling pathways are not entirely understood. With androgenic and estrogenic compounds only somewhat identified with their physiological effects on different systems, such as the muscular and endocrine systems, there are only postulations regarding the direct and indirect physiological interactions of growth-promoting implant strategies on the digestive system.

Hormone and Gut Relationship

Research involving the gut and associated microbial communities has been developing within the past years for mammalian studies to characterize microbial communities. In order to further understand how the gut microbiome can affect growth, health and disease, or other physiological systems, studies have been conducted to analyze hormones interaction with the gut microbiome. For the purpose of this study, hormones identified are ones associated with implant strategies, i.e. estrogen and testosterone influences. Human studies have determined estrogen in association with changes in the gut microbiome. Estrogenic receptors have been identified on gut tissues in monogastric species, while androgenic receptors have not yet been identified in research. Further, the presence of the hormones themselves aid to alter diversity in the gut microbiome. There is potential for the introduction of implant strategies to the system to alter certain rumen microbiome variability.

Estrogen and Gut Relationships

To identify potential effects of implanting strategies on the gut microbiome, it is important to identify potential receptors on the gut to influence metabolic responses. Studies have detailed the importance of the gut microbiome on estrogen levels present in human systems. The gut microbiome impacts E₂ levels in the host by secreting β -glucuronidase, which is an enzyme that deconjugates E₂ to allow binding to E₂ receptors that lead to downstream physiological effects (Plottel and Blaser, 2011). Further, a decrease in the diversity of the gut microbiome in humans results in a decrease in β -glucuronidase activity, which can alter metabolic activity (Baker et al., 2017). Thus, estrogenic activity in the gut can influence the microbiome. In rats, addition of E₂ allowed tissues to improve responses when exposed to intestinal perfusions, indicating that there are receptors on GI tissues for E₂ (Doucet et al., 2010). The presence of these receptors indicates that estrogen does have an impact on the gut, with potential of metabolic activities to alter the gut microbiome and other physiological activity. While this has been well-established in monogastric species, there is still speculation on metabolic pathways altered by estrogen in the rumen.

Estrogen-related receptor α [ESRRA] has been identified on the surface of rumen tissue in calves, assisting in development of the rumen (Connor et al., 2014). This DNA transcription factor, otherwise known as nuclear protein receptors, binds to metabolites to alter transcription of genes. While present in the rumen tissue during weaning, these receptors may remain after rumen development. Along with that, they may be associated with certain growth characteristics in the rumen. Thus, with administration of implanting strategies that contain estrogenic compounds, there is a potential for them to influence the gut microbiome and other physiological downstream processes via receptors present on gut tissues.

Testosterone and Gut Relationships

In human studies, testosterone production has been demonstrated in the gut by *Clostridium scindens*, converting glucocorticoids to androgens through enzymatic activity (Ridlon et al., 2013). As previously mentioned, in animals implanted with androgenic compounds, even in combination with estrogenic compounds, there is typically a decrease in glucocorticoids present in the system. This microbial community is associated for degrading bile salts and participating in lipid digestion in humans. In ruminants, *Clostridium* primarily play a role in cellulose degradation; however, *Clostridium scindens* has not been identified in the gut microbiome of ruminants. Testosterone receptors on gut tissues have not been identified in either monogastric or ruminant animals and future research is needed towards identifying how these hormone receptors may play an important role in the digestive system. However, there may be microbial communities present in the gut microbiome that play an important role in the regulation of testosterone circulation.

The Rumen Microbiome

The GI tract in vertebrates and mammals is home to a diverse and rich microbial population that aids in digestion. These microbial communities play an important role in host health and physiology, altering their ability to utilize energy sources and handle disease states. The rumen is a ruminant's primary source of digestion, being an anaerobic and pH-balanced environment that serves as a unique tissue to these animals (Hungate, 1960). Due to a ruminant's reliance on their gut microbiome to digest and provide energy to the animal, many studies look towards linking the rumen microbiome to feed efficiency and growth metrics. The rumen microbiome consists of bacterial, archaeal, protozoal, and fungal communities. Bacterial communities in the rumen make up the majority of the total microbial population, with archaea making up approximately 0.3-4% of rumen biomass and function as anaerobic methanogens (Lin et al., 1997), protozoa comprising typically under half of the microbial communities (Hungate, 2013, Newbold et al., 2015), and fungi estimated to be between 8-20% of rumen microbial biomass (Rezaeian et al., 2004). Importantly, many studies looking at the rumen microbiome primarily focus on the bacterial communities rather than the microbiome as a whole. Therefore, more research is required regarding all kingdoms present in the rumen environment.

Ruminal Bacterial Communities

Metagenomic approaches to analysis of 16S genes have rapidly improved within the last decade, such as the usage of next-generation sequencing. Using advanced technology, multiple studies have been performed on the rumen

bacterial communities in cattle, assessing different production factors such as feed efficiency (Carberry et al., 2012, Myer et al., 2015) and milk yield (Jami et al., 2014). In order to understand how feed efficiency can be altered by presence of bacterial communities in the rumen, further research is necessary to see how they contribute to the host growth and health.

Studies have identified that the majority of bacterial communities in the rumen consist of Bacteroidetes and Firmicutes, making up approximately 70-80% of the ruminal microbial communities (McCann et al., 2014, Myer et al., 2015). These communities can shift depending on availability of certain feedstuffs, notably seen as an increase in Firmicutes in the presence of forage-based diets. The ratio present of Bacteroidetes to Firmicutes has been noted in studies to potentially have an effect on digestibility of nutrients and typically correlated with lipids present in the diet (Jami et al., 2014). In human studies, when Firmicutes dominate the Bacteroidetes:Firmicutes ratio, higher rates of obesity are found (Koliada et al., 2017). Importantly, this ratio is of interest but may have little value behind it, thus not typically a target in animal research. While this is suspected to contribute towards feed efficiency in cattle, more research is required to fully understand how the variation of phyla alters feed efficiency.

When analyzing steers differing in feed efficiency, Myer et al. found significant differences in relative abundances of bacterial communities. Highly feed efficient animals, identified as low daily gain and high daily feed intake and thus more efficient, contained genera that differed from animals that were not feed efficient, containing namely *Ruminococcus*, *Lactobacillus*, and *Succiniclaticum* (Myer et al., 2015). The genus *Prevotella* has been noted in studies to be linked to animals that are less efficient (Carberry et al., 2012), but more research is required to truly define a correlation to bacterial communities and feed efficiency. While *Prevotella* is linked to low-RFI animals, it is reportedly the most abundant genus present in the rumen microbiome (Stevenson and Weimer, 2007, Pitta et al., 2010).

With the knowledge that ruminal bacterial communities can play a role in altering feed efficiency, further research is needed to identify the linkage between microbial communities and genetics and management styles.

Ruminal Archaeal Communities

Archaeal communities present in the rumen microbiome have been well-characterized for their role in methanogenesis, which ultimately contributes to methane [CH₄] emissions from ruminants. Importantly, this microbial population is often targeted when addressing environmental footprints associated with agriculture; however, presence of archaeal communities has a weak correlation to methane emissions from animals (Tapio et al., 2017). When assessing energy sources to ruminants, archaeal populations are often analyzed in an attempt to

reduce ruminal CH₄, as this is a source of loss of energy for the host (Johnson and Johnson, 1995). Johnson et al. predicted this loss to be anywhere from 2 to 12% of gross energy intake, which could otherwise be a source to the host to increase production-relevant factors such as growth or milk production. Thus, extensive studies have been conducted to analyze how to adjust archaeal populations to more effectively harness energy in ruminants.

Many factors have been found to influence methane production and emissions in cattle, such as levels of feed intake, feed processing, and presence of lipids or ionophores in the diet. While studies have adjusted levels of CH₄ emissions, it is important to note that it is energetically favorable for the rumen to convert to CH₄, oftentimes making different pathways readily occur (Janssen and Kirs, 2008). Interestingly, these factors primarily adjust precursors to methanogenic activity, overall reducing total available of formate and H₂. Ionophores added to the diet have been proven to decrease cellulolytic and amylolytic bacteria, such as *Butyrivibrio fibrisolvens* and *Streptococcus bovis* (Russell and Strobel, 1989). During this activity, there is a shift in VFAs, typically seen as a large increase of propionate and a decrease of acetate (Wallace et al., 1980). Importantly, this decrease in acetate allows for more feed energy to be available to the host, as propionate is more energetically favorable for oxidation. Along with addition of ionophores to the diet, increasing forage digestibility also mitigates much of CH₄ emissions through changes in the archaea population (Hristov et al., 2013). Notably, these shifts in bacterial communities can then adjust variation of archaeal communities. Thus, methane production from beef cattle is more likely to be associated with the presence of certain archaeal communities in the rumen rather than the overall abundance of archaea.

There are two primary pathways for methanogenesis in the rumen, both requiring and utilizing archaea. The hydrogenotrophic pathway functions as to convert H₂ and CO₂, byproducts of protozoal, bacterial, and fungal groups, to CH₄ (Hungate, 1967, Martin et al., 2010). The addition of H₂ + CO₂ is assumed to be the equivalent of formate, which can be utilized by all archaea for CH₄ production (Janssen and Kirs, 2008). This metabolic activity is crucial to rumen functionality, as presence of H₂ can result in an inhibition of hydrogenase activity and limit oxidation of sugars (McAllister and Newbold, 2008). Without these functions, acidosis events can occur in the rumen, in which low pH in the rumen due to hydrogen ions will increase the enzymatic activity of lactate dehydrogenase, increasing conversion of pyruvate to lactate (Russell and Hino, 1985). Ultimately, this makes normal ruminal environments harder to recover from the acidotic state (Owens et al., 1998).

The primary source of hydrogenotrophic archaea stems from the genus *Methanobrevibacter*, which is divided into two clades: SGMT clade and the other [RO] clade (Kittelmann et al., 2013). Minor hydrogenotrophic archaea genera include *Methanosphaera*, *Methanimicrococcus*, and *Methanobacterium*.

Interestingly, previous studies have identified positive correlations with *Methanobrevibacter ruminantium* clade and bacteria in the family of *Fibrobacteraceae*, as well as *Ruminococcaceae* (Kittelmann et al., 2013). These bacterial communities may contribute to the methanogenic population due to being primary cellulolytic bacterium present in the rumen (Kobayashi et al., 2008). Bacteria within the *Ruminococcus* spp. are known for their production of H₂, while *Fibrobacter* spp. have been identified to produce formate (Leahy et al., 2010). As previously mentioned, both of these are utilized in methanogenic activity to produce methane (Hungate, 1967, Balch et al., 1979, Martin et al., 2010). Current research is still working towards identifying true differences and associations between archaeal species present in the *Methanobrevibacter* genus and how it relates to hydrogenotrophic activity in the rumen, subsequently affecting CH₄ production.

The second pathway for methanogenesis from archaeal communities and providing the majority of substrate for the process is methyl groups, found in forms of methylamines and methanol in the rumen (Neill et al., 1978). The primary source of methylamines in the diet are from plant membranes, which are degraded by rumen microorganisms from choline methyl groups to trimethylamine to produce free choline and methane (Dawson et al., 1974, Neill et al., 1978). Methanol primarily is derived from plant polysaccharides, which undergo hydrolysis of methanolic side-groups (Tapio et al., 2017). These methanogenic mechanisms occurring by archaeal communities ultimately remove H₂ ions and utilize formate to act as a normally functioning rumen environment.

Ruminal Protozoal Communities

Despite the knowledge that protozoa can reach up to 50%, the role of protozoa in the rumen remains relatively unknown (Williams and Coleman, 1997, Koenig et al., 2000). Protozoal communities present in the rumen appear to have a strikingly large role in maintenance of bacterial communities as well as playing a role in methanogenesis (Newbold et al., 2015). Rumen protozoa also function to digest multiple bacteria present, however more research is needed to determine the exact function of this activity (Williams and Coleman, 1997, Hungate, 2013). Historically, protozoa were assumed to play a part in host health and wellbeing, yet current research is revealing some of their purpose in the rumen environment. Current research focuses primarily in the rumen of sheep and lesser studies available in cattle.

The entire removal of protozoal communities from the rumen has proven to have little impact on animal welfare (Williams and Coleman, 1997, Belanche et al., 2011, Zhou et al., 2011). Digestibility of fibrous materials, namely ADF and NDF, decreases in presence of less protozoa, potentially due to fibrolytic activity by protozoal communities. Without ciliate protozoa present in the rumen, microbial

protein supply increases up to 30% and reduces CH₄ production by up to 11%. Potentially, the decrease of the protozoa population in the rumen can result in more energy available to the host, as there would be less methane emissions due to a decrease of excess urea and ammonia levels (Koenig et al., 2000, Hristov et al., 2013, Newbold et al., 2015). However, due to the unique membranes of protozoal communities, functionality is often hard to pinpoint.

Recent research has identified two main groups of protozoa, identified as holotrich ciliate and entodiniomorphids (Belanche et al., 2011). The primary holotrich protozoa that have been identified and studied are *Isotricha intestinalis*, *Isotricha prostoma*, and *Dasytricha ruminantium* (Williams and Coleman, 1997). Holotrich species are widely dependent on environment and nutritional status, as well as frequency of feedings (Williams and Coleman, 1997). These protozoa increase in prevalence when the animals are being fed diets high in soluble carbohydrates (Clarke, 1965). Primary members of the protozoal population in ruminants are *Epidinium*, *Polyplastron*, and *Entodinium*, associated with entodiniomorphids, most commonly known for their ability to engulf and digest bacteria (Williams and Coleman, 1997, Kamra, 2005). Namely, *Epidinium* and *Polyplastron* appear to have endoglucanase and xylanase activity, working to break down cellulose and hemicellulose (Williams and Coleman, 1997). Smaller groups of *Dasytricha* perform glucosidase and cellobiosidase activity, while having insignificant fibrolytic activity (Takenaka et al., 2004). Further research is required in this field to identify protozoal communities present in the rumen of cattle.

Volatile Fatty Acids

Studies regarding VFAs in ruminants has been extensively studied to understand their functionality in the rumen. Energy requirements are often met in ruminants through the absorption of short-chain fatty acids through the rumen wall, allowing for analysis of VFAs to serve as an indicator of energy utilization (Cottyn et al., 1968). The primary VFAs in ruminants are acetic, propionic, and butyric acid, which are all products of fermentation of organic matter via microbial communities in the gut. These VFAs have been proven to effect production-relevant parameters such as growth (Dijkstra, 1994). Importantly, the presence and levels of VFAs are highly dependent on the feedstuffs available to animal (Bergman, 1990). In diets that are high in grain, an increase in propionate is typically seen, balanced by a decrease in acetate (Tajima et al., 2000). This also is associated with changes in methane emissions, as previously mentioned.

In order to understand energy utilization in the rumen, VFAs have been linked to different feed efficiency metrics to detail which VFAs are present in high- and low-RFI animals. Specifically, low-RFI animals have been noted to have more butyrate and acetate than high-RFI animals (Guan et al., 2008). Other studies have been performed to attempt to characterize VFAs for their role in feed efficiency, however

more research is required to understand how feed efficiency and VFAs could be linked. These VFAs play an important role in metabolic function by generating ATP for different pathways (Seymour et al., 2005). Characterizing metabolic activity in the rumen may lead to an understanding of factors affecting feed efficiency via the gut environment.

Rumen Metabolites Associated with Gain

Metabolomics aims to characterize metabolite profiles associated with biological samples, such as serum, plasma, or rumen. This knowledge contributes towards the development of research to understand genomic importance of metabolites as well as understanding certain production-relevant parameters in livestock. Analytical chemistry techniques, such as mass spectrometry, high-performance liquid-phase chromatography, and nuclear magnetic resonance have improved in the past years, allowing for this field to grow in popularity. Along with that, hundreds to thousands of metabolites can be detected within even one sample for relatively low costs (Zhang et al., 2012). Targeted or untargeted metabolomics is used to identify the metabolome, with untargeted metabolomics being primarily used in newer fields to characterize unknown metabolites that may not have been previously identified.

In livestock research, targeted and untargeted metabolomics have been used on serum, plasma, and milk to link certain desirable phenotypic traits to the metabolome (Goldansaz et al., 2017). Interestingly, multiple metabolomics studies have been used to associate genomics with production-relevant features, such as RFI in beef cattle through measurements of metabolites in serum and plasma (Karisa et al., 2014, Artegoitia et al., 2017, Clemmons et al., 2017) or milk production in dairy cattle (Lu et al., 2013). More recently, metabolomics has been used to characterize metabolites present in rumen fluid in dairy cattle (Ametaj et al., 2010), which can be used to differentiate between metabolomes in animals presented with different feedstuffs. With the knowledge that feed intake and nutrition can alter the rumen microbiome, studies have begun to analyze how feed efficiency and growth parameters may also adjust the rumen metabolome (Artegoitia et al., 2017).

The use of untargeted metabolomics has provided some insight into the metabolome of rumen fluid. Since serum and plasma metabolomes in cattle have been more deeply characterized than the rumen fluid metabolome, there is a need to expand research into the metabolites present in rumen fluid. Importantly, metabolic activity in the rumen may be an indicator of microbial function or metabolic capability in the rumen. Multiple studies have been performed to begin to characterize metabolites present in rumen fluid to associate with certain

feedstuffs (Saleem et al., 2013) and desirable production traits (Artegoitia et al., 2017). With the knowledge previously mentioned that changes in ruminal microbial communities are associated with differences in feed efficiency in beef cattle (Myer et al., 2015), analysis of metabolites is necessary to understand the changes in metabolism due to these differences.

Primarily, rumen fluid has been previously characterized to contain many products of microbial fermentation, such as VFAs, phospholipids, and different esters (Saleem et al., 2013). Artegoitia et al. further detailed the rumen metabolome, identifying linoleic and alpha-linolenic metabolic pathways as significantly impacting metabolic pathways in rumen fluid that were highly associated with ADG in steers. Along with that, biosynthesis of aromatic amino acid was also altered when analyzing differences between steers different ADG (Artegoitia et al., 2017). It is important to note that biological pathways directly associated with ADG have not been identified; however, metabolomics research can work towards identifying metabolic activity that may link to more efficient animals. Further identification of metabolites present in rumen fluid with differing ADG as well as diets can lead to a greater understanding of metabolic activity in the rumen environment.

Conclusions

With the need for an increase in sustainably-produced beef, researchers and producers are aiming to improve feed efficiency in cattle. Feed efficiency can be altered by multiple factors, thus making it hard to target. Availability of nutrients, genetics, management strategies, and the ruminal environment can overall alter feed efficiency in cattle. Thus, research needs to identify the overlap in these factors to understand how to create the most efficient animal. With the presence of hormone receptors on physiological systems, namely the GI tract, there is potential for implant strategies to influence the rumen microbiome, leading to an increase in weight gain and feed efficiency. Current research does not analyze how the rumen microbiome and metabolome can be affected by implanting strategies, leaving a crucial gap to be filled in livestock research.

CHAPTER TWO

EFFECTS OF A MODERATE AND AGGRESSIVE IMPLANT STRATEGY ON THE RUMEN MICROBIAL COMMUNITIES AND METABOLOME IN STEERS

Abstract

The effects of growth-promoting implants have been well-defined for their ability to impact growth performance in beef cattle. Production-relevant microbiomes in the rumen have also been associated with growth traits. However, the role of implants on the rumen microbiome is not understood. The objective was to determine if varying doses of implant hormones cause gain-associated ruminal microbial community shifts. To assess this, a completely randomized design was used and 336 fall-born steers 450 to 470 days of age from the germplasm evaluation population at the US Meat Animal Research Center (Clay Center, NE) were divided into two treatment groups: 1) a moderate implant strategy of Revalor-IS (80 mg trenbolone acetate and 16 mg estradiol) followed by Revalor-S (120 mg trenbolone acetate and 24 mg estradiol) and 2) an aggressive implant strategy of Revalor-IS followed by Revalor-200 (200 mg trenbolone acetate and 20 mg estradiol). Steers were fed the same diet (57.0% dry-rolled corn, 30% wet distiller's grains with solubles, 8.0% alfalfa hay, 4.25% supplement, and 0.75% urea, on a DM basis). Body weights were collected once per month with initial weights of 439.8 ± 43.1 kg. After implants were administered for 84 days, rumen content was collected via orogastric tubing. Samples were sequenced targeting bacterial V1-V3 16S rRNA gene regions, V3-V4 for archaea, and partial 18S rRNA gene of protozoa. Sequences were processed in R utilizing phyloseq with DADA2 and analyzed with DESeq2 to test differential abundances. Untargeted metabolomics were performed on rumen fluid using the UHPLC-HRMS system. Production data between implant strategies was analyzed using a mixed model ANOVA (SASv9.4, Cary, NC) followed by analysis of least square means. Alpha- and beta-diversity between strategies did not differ for bacteria, archaea, or protozoa ($P > 0.05$). Average daily gain was different ($P = 0.01$; 1.72 vs 1.66 ± 0.02 kg, aggressive vs moderate, respectively); however, large microbial community shifts were not associated implant strategy. Two metabolites, acetyllysine and N-acetylorithine, were significant between implant strategy ($P \leq 0.04$). Understanding associations between the rumen microbiome and implant strategies may allow improvement of growth in beef cattle.

Introduction

The effects of growth-promoting implants have been well-defined for their improvement of growth performance in beef cattle. These implants not only increase average daily gain [ADG], but also can adjust feed efficiency metrics. Implants have been proven to increase feed efficiency by 15% and ADG by 30%, ultimately serving as one of the most efficient ways to improve feedlot operations (Duckett et al., 1996, Preston, 1999). In addition, implant strategies can be categorized by their ability to affect growth. The amount of growth-promoting hormones dictate the extent to which growth is influenced. Costs of these implants can vary depending on the levels of estrogenic or androgenic hormones present, however typically are between \$1 to \$3 per head (Mader, 1994). Historically, cattle remained in feedlot operations for around 100 days, but more recently are in feedlots for 200 days or more to maximize growth potential (Smith et al., 2018). Growth-promoting implant strategies often involve re-implanting animals to cause additive gain, thus keeping animals in feedlots longer. Using re-implantation strategies allow producers to see a gain response increase of anywhere between 5-20%, making these strategies crucial to feedlot operations (Preston, 1999).

While multiple implants are available on the market that vary in amounts of androgenic and estrogenic hormones present, Revalor implants primarily consist of a synthetic testosterone called trenbolone acetate [TBA] and natural estradiol [E₂]. The mechanisms by which TBA and E₂ directly and indirectly alter weight gain and feed efficiency in beef cattle are still being elucidated; however, studies have been conducted on sera metabolites (Johnson et al., 1996b, Smith et al., 2018), muscle tissue (Roeber et al., 2000), and transcriptomes of the muscle and liver in order to understand effects on growth (Elgendy et al., 2016). Studies have identified the importance of estrogenic and androgenic compounds on abundances of insulin-like growth factor I [IGF-I] and growth hormones [GH] (Preston, 1975, Johnson et al., 1996b). Additionally, androgenic compounds have been demonstrated to directly alter muscle cell proliferation, while estrogenic compounds have indirect effects on metabolites and transcriptomes of the liver and muscle to improve gain and feed efficiency (Elgendy et al., 2016).

Further, these hormones could be interacting with different receptors present on other tissues, namely the gastrointestinal tract. Estrogen-like receptor- α has been identified on ruminal tissue in calves (Connor et al., 2014), potentially indicating steroidal activity on the rumen and thus the microbial communities present in the rumen. In humans, bacterial communities have been demonstrated to cause shifts in estrogenic activity, showing interaction between the gut microbiome and hormones (Plottel and Blaser, 2011, Baker et al., 2017). While androgenic receptors have not been identified on the gut, there is potential for the presence of testosterone-related substances to alter metabolic activity and therefore have an influence on the gut microbiome. Metabolic pathways are altered through the use

of growth-promoting hormones, thus could play a role in altering the rumen microbiome between moderate and aggressive implant strategies.

The rumen microbiome has been linked in previous studies to impact weight gain and feed efficiency (Mizrahi, 2011, Myer et al., 2015). Interestingly, studies conducted have found shifts in microbial communities between animals varying in ADG and feed intake (Myer et al., 2015). At the bacterial genus level, many *Prevotella* have been identified in cattle that are feed efficient (Myer et al., 2015). In addition, studies have attempted to identify archaeal and protozoal communities present in the rumen that may be influencing weight gain. Many archaea play an important role in methane emissions in cattle (Janssen and Kirs, 2008), such as genera *Methanobrevibacter* and *Methanobacterium* (Kittelman et al., 2013). Importantly, archaeal communities producing methane result in a loss in energy provided to the host; thus, targeting these communities to reduce methane emissions as well as improve energy resources to the host could improve gain in beef cattle (Johnson and Johnson, 1995). Research to improve beef cattle efficiency also focuses on N utilization, as low N utilization results in decreased productivity in a beef cattle operation. Protozoal communities have been considered major contributors to intraruminal N cycling (Jouany, 2003), overall reducing host protein utilization and muscle growth in beef cattle. Fermentation by microbial communities in the rumen affect nutrient breakdown and volatile fatty acid [VFA] synthesis (Firkins et al., 2006). These VFAs contribute 70% or more of energy to the host, thus are an important factor in animal growth. Microbial communities and fermentative products present in the metabolome may be associated with host phenotypes for feed efficiency and weight gain in beef cattle.

Fermentation occurring by microbial communities in the rumen produce metabolites that can be identified through the use of untargeted metabolomics in an attempt to characterize desirable phenotypic traits, such as feed efficiency and growth in beef cattle (Goldansaz et al., 2017). While metabolic profiles have been characterized in serum, plasma, and milk, rumen fluid is less commonly analyzed. Direct correlations between blood and rumen metabolites cannot be assumed, therefore prompting further research of the rumen metabolome (Clemmons et al., 2020). Previous studies have acknowledged the importance of metabolites associated with linoleic pathways in rumen fluid, as they are highly associated with ADG in steers (Artegoitia et al., 2017). Importantly, the variation in these pathways moderating nutrient use may reflect how the fermentative and metabolic capabilities of microbial communities are altering animal efficiency. Thus, there is a need to examine rumen metabolites that could be due to host metabolic activity or resulting from fermentation output from microbial communities. The objective of this study was to identify potential associations with growth-promoting hormones and their influence on the rumen microbiome and metabolome between moderate and aggressive implant strategies to augment gain in beef cattle.

Methods and Materials

Animal Use Ethics Statement

The U.S. Meat Animal Research Center [MARC] Animal Care and Use Committee approved procedures utilized on animals in this study (Experiment Number: 5428-31000-092-06).

Experimental Design

Using a completely randomized design, germplasm evaluation steers (T population code) aging 450-470 days of age from U.S. MARC ($n = 336$) were placed into feedlot pens. These steers consist of the top 18 cattle breeds in the U.S. Steers were given *ad libitum* access to water and gradually adjusted to a feedlot diet consisting of 87% concentrate (Table 2.1). Non-shrunk body weight was then measured once a month throughout the entire study with an average starting body weight of 439.8 ± 43.1 kg.

Table 2.1. Diet on a dry-matter basis

Feedstuff	% Inclusion of Feedstuff
Dry-rolled corn	57.0
Wet distillers' grains with solubles	30.0
Alfalfa hay	8.0
Vitamin and mineral supplement with Rumensin and Tylan	4.25
Urea	0.75

Treatments were randomly assigned to steers, being either a moderate implant strategy or an aggressive implant strategy. The moderate implant strategy ($n = 167$) consisted of an initial implant of Revalor-IS (80 mg of trenbolone acetate and 16 mg of estradiol) followed by a treatment re-implantation of Revalor-S (120 mg of trenbolone acetate and 24 mg of estradiol) (Merck Animal Health, Summitt, NJ, USA). The aggressive implant strategy ($n = 169$) consisted of an initial implant strategy of Revalor-IS, followed by a treatment re-implantation of Revalor-200 (200 mg of trenbolone acetate and 20 mg of estradiol). The initial implant was administered after 60 days of adjustment to the high-concentrate diet, with the re-implantation of the moderate or aggressive implant administered 90 days after the initial implant of Revalor-IS.

Rumen Sampling from Steers

After 85 days past the treatment implant of either Revalor-S or Revalor-200, 200 ml of rumen fluid was collected from each animal via orogastric tubing. A ½-inch anhydrous tube was connected to a vacuum flask and vacuum pumped to collect fiber and fluid. Tubes were discarded between animals to prevent cross-contamination. Ruminal fluid from individual animals was separated into 50 ml conical tubes and snap-frozen in liquid nitrogen. Samples were then stored at -80°C until further analysis.

DNA Extraction and Purification for Microbial Communities

Rumen samples containing fiber and fluid were sent to the University of Tennessee, Knoxville, TN for DNA extraction and purification, following the rumen digesta extraction protocol (Yu and Morrison, 2004). From the 50 ml conical tubes, 0.2 g of fresh sample with fiber was added to a 2 ml beaded screw cap tube containing 0.5 mm ZR BashingBead lysis matrix (Zymo Research, Irvine, CA, USA) for cell lysis. After addition of sample, 1 ml of lysis buffer (500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, and 4% sodium dodecyl sulfate [SDS]) The samples were then homogenized for 3 minutes at 21 Hz and incubated at 70°C for 15 minutes, with inversions every 5 minutes. Samples were then centrifuged at 4°C for 5 minutes at 16,000x g. Supernatants were then individually transferred to a fresh 2-ml tube. Following, an additional 300 µL of lysis buffer was added to the lysis tube and steps were repeated. The supernatant was then pooled for individual samples.

Precipitation of nucleic acids was performed by adding 260 µL of 10-M ammonium acetate to each lysate tube. Samples were mixed well and incubated on ice for 5 minutes. After ice, the tubes were then centrifuged at 4°C for 10 minutes at 16,000x g. The supernatant was split into two 1.5-ml tubes, and one volume of isopropanol was added and mixed well. Isopropanol precipitation occurred after samples were left on ice for 30 minutes. Samples were centrifuged at 4°C for 15 minutes at 16,000x g and the resulting supernatant was discarded, leaving a pellet in the bottom of the tubes for each sample. The nucleic acid pellet was washed with 70% ethanol and dried under vacuum for 3 minutes. Once dried, the nucleic acid pellet was dissolved in 100 µL of Tris-EDTA [TE] buffer and the separated aliquots were pooled for individual samples.

Following precipitation of nucleic acids, the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA, USA) was utilized for purification. Any contaminants of RNA were then removed by adding 2 µL of DNase-free RNase (10 mg/ml) and incubated at 37°C for 15 minutes. Then, to remove protein contamination, 15 µL of proteinase K and 200 µL of Buffer AL were added, mixed well, and incubated at 70°C for 10 minutes. Following incubation, 200 µL of 100% ethanol was added and mixed well. Samples were transferred to QIAamp columns (QIAGEN, Valencia, CA, USA) and

centrifuged at 16,000x g for 1 minute. Flow through was discarded and this process was repeated, adding Buffer AW1 and centrifuging, and then Buffer AW2 and centrifuging under the same conditions. The column was then dried by centrifugation at room temperature for 1 minute. Following drying, 70 µL of Buffer AE was added to the column membrane and then the samples were incubated at room temperature for 2 minutes. Then, 30 µL of Buffer AE was added to the column membrane and incubated under the same conditions. To elute DNA, the QIAamp column was placed into a 1.5-ml tube and centrifuged for 1 minute. The DNA samples were stored at -20°C until ready for amplification and library preparation.

DNA Amplification of Microbial Communities

Primers used for DNA amplification are listed in Table 2.2. Amplicon libraries of the 16S rRNA gene for bacteria (V1-V3) were prepared as described (Kozich et al., 2013), using primers 27F (Stahl, 1991) and 519R (Lane et al., 1985). Each 20 µL polymerase chain reaction [PCR] amplification reaction contained 0.5 µL Terra PCR Direct Polymerase Mix (0.625 Units), 7.5 µL nuclease-free sterile water, 10 µL 2X Terra PCR Direct Buffer, 1 µL indexed fusion primers (10 µM), and 1 µL DNA (20 to 70 ng). Thermocycling conditions were as follows: initial denaturation at 98°C for 3 minutes, 25 cycles at 98°C for 30 seconds, 55°C for 30 seconds, and 68°C for 45 seconds, and a final extension of 68°C for 4 minutes.

For archaeal communities, amplicon libraries of the 16S rRNA gene for archaea (V3-V4) were prepared using primers ARC344F_TS (Wemheuer et al., 2012) and ARCH806R_TS (Takai and Horikoshi, 2000), found in Table 2.2. Each 10 µL run contained 4.2 µL nuclease-free sterile water, 1.25 µL 10X Buffer, 0.65 µL 25 mM MgCl₂, 0.2 µL 25 mM dNTPs, 0.5 µL FC1:TS1 primer (4 µM), 0.2 µL HotStar Taq (QIAGEN, Valencia, CA, USA), 1 µL of bacterial indexes (1.875 µM), 1 µL of methanogen primers (1 µM), and 1 µL of DNA (15 ng/µL). Thermocycler conditions were as follows: 95°C for 15 minutes, 8 cycles of 94°C for 1 minute, 55°C for 30 seconds, and 72°C for 45 seconds, 24 cycles of 94°C for 20 seconds, 62°C for 30 seconds, and 72°C for 30 seconds, and a final extension of 72°C for 5 minutes.

Amplicon libraries of the 18S rRNA gene for protozoa (V3-V4) were prepared using primers P-SSU-316F (Sylvester et al., 2004) and GIC758R (Ishaq and Wright, 2014) (Table 2.2). Each 10 µL reaction consisted of 3.2 µL nuclease-free sterile water, 1 µL 10X Buffer, 0.65 µL 25 mM MgCl₂, 0.2 µL 25 mM dNTPs, 1 µL protozoal primers (0.5 µM), 0.5 µL FC1:TS1 primer (4 µM), 0.2 µL HotStar Taq (QIAGEN, Valencia, CA, USA), 1 µL barcode, and 1 µL DNA (15 ng/µL). Thermocycler conditions were as follows: 95°C for 15 minutes, 8 cycles of 94°C for 1 min, 50°C for 30 seconds, and 72°C for 45 seconds, and 24 cycles of 94°C for 20 seconds, 62°C for 30 seconds, and 72°C for 30 seconds, and a final extension of 72°C for 5 minutes.

Table 2.2. Primers for DNA PCR amplification

	Regions	Primers	Sequences	Source
Bacteria	V1-V3	27F	KRGTTYGATYNTG GCTCAG	(Stahl, 1991)
		519R	GWRTTACCGCGG CKGCTG	(Lane et al., 1985)
Archaea	V3-V4	ARC344 F_TS	ACGGGGYGCGCA GGCGCGA	(Wemheuer et al., 2012)
		ARCH80 6R_TS	GGACTACVSGGGT ATCTAAT	(Takai and Horikoshi, 2000)
Protozoa	V3-V4	P-SSU- 316F	GCTTTCGWTGGTA GTGTATT	(Sylvester et al., 2004)
		GIC758 R	CAACTGTCTCTATK AAYCG	(Ishaq and Wright, 2014)

After amplification, PCR products from each sample were normalized (1 to 2 ng/ μ L) using the Just-a-Plate™ 96 PCR Purification and Normalization kit (Charm Biotech, MO, USA) as described by the manufacturer. The normalized libraries were pooled (10 μ L/sample) and purified using the Nucleospin® Gel and PCR Cleanup kit (Takara Bio USA, Inc., Mountain View, CA) according to manufacturer's protocol. Quality control was performed on libraries using the BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and quantified using the DeNovix QFX Fluorometer (DeNovix dsDNA Fluorescence Quantification Assay). The libraries were sequenced using the 2x250, v2 500-cycle kit and the Illumina Miseq System (Illumina, San Diego, CA, USA).

DNA Amplicon Sequence Data Processing for Microbial Communities

The Illumina 2x250 fastq sequencing files for bacterial and archaeal communities were processed through a R pipeline as described by (Callahan et al., 2016b). Fastq Illumina files were entered into R and open-source R packages 'phyloseq' (McMurdie and Holmes, 2013) and 'DADA2' (Callahan et al., 2016a) were utilized to perform filtering, merging, and taxonomic assignment. Forward and reverse reads were trimmed based on quality score ($Q \geq 25$) and expected errors per read were set to two for forward reads and four for reverse reads, filtering out any data

not meeting these criteria (Edgar and Flyvbjerg, 2015). Following, Divisive Amplicon Denoising Algorithm 2 [DADA2] was used to more accurately identify real variants and correct for amplicon errors using a naïve Bayesian classifier (Wang et al., 2007, Callahan et al., 2016a). From DADA2, amplicon sequence variants [ASVs] were generated, which can moderately increase genetic resolution in comparison to 97% operational taxonomic units [OTUs] (Callahan et al., 2017, Glassman and Martiny, 2018). Quality-filtered forward and reverse reads were then merged and chimeras were removed. Sequences underwent taxonomic assignment at the genus level using the SILVA v132 database (Quast et al., 2012). The singletons, *Cyanobacteria*, and Protozoa were removed from the data. For bacterial communities, Archaea was removed as well, and for archaeal communities, Bacteria was removed.

Protozoal fastq sequencing files were also processed through the same pipeline as described by (Callahan et al., 2016b) and utilized the same open-source R packages 'phyloseq' and 'DADA2'. Forward and reverse reads were trimmed based on quality score ($Q \geq 25$) and expected errors per read were set to two for forward and five for reverse reads, filtering out data not meeting criteria. Importantly, samples with less than 10,000 reads following filtering protocol were removed. Then, DADA2 was used to identify ASVs, forward and reverse reads were merged, and chimeras were removed. Sequences underwent taxonomic assignment at the genus level using the SILVA v132 database, and singletons, *Cyanobacteria*, Archaea, and Bacteria were removed from data.

Rumen Metabolite Extraction

A 2 mL aliquot of rumen fluid was transferred to two sterile 2 mL microcentrifuge tubes and centrifuged at 6,000x *g* for 15 minutes at 4°C. Supernatant was aspirated and combined from each tube using a 3 mL syringe, and then filtered through a 0.22 µm syringe filter (Midsco, St. Louis, MO, USA) to a sterile 2 mL cryovial. Samples were stored at -20°C until further analysis.

Water-soluble metabolites were extracted from 100 µL of rumen fluid using 4:4:2 acetonitrile/methanol/water with 0.1 M formic acid, as previously described (Burke et al., 2019). Following extraction, the supernatant was dried under N₂ then resuspended in 300 µL of water. All solvents used were HPLC grade, purchased from Fisher Scientific (Hampton, NH, USA). Metabolites were identified using ultra-high-performance liquid chromatography high resolution mass spectrometry (UHPLC-HRMS) (Thermo Scientific, San Jose, CA, USA), with a previously validated untargeted metabolomics method (Lu et al., 2010). Prior to mass analysis, reverse-phase (RP), ion-pairing chromatography was used to separate metabolites based on polarity. A Synergi Hydro RP column (100 mm x 2.1 mm, 2.6 µm, 100 Å) and an UltiMate 3000 pump (Thermo Fisher) were used for

chromatographic separation. An Exactive Plus Orbitrap MS (Thermo Fisher) was coupled to the UHPLC system and used for mass analysis.

Statistical Analyses

For 16S and 18S processed data through R, alpha- and beta-diversity metrics were analyzed. For alpha diversity, observed communities was calculated alongside expected (Chao1) as well as richness and diversity estimates were performed (Shannon). Alpha diversity was calculated individually for bacteria, archaea, and protozoa and differences between treatments were tested in SAS v9.4 (SAS Institute, Cary, NC, USA) using a mixed model analysis of variance with least square means, with significance determined by $\alpha = 0.05$. Beta diversity was measured using a Bray-Curtis distance matrix producing a principal coordinates analysis [PCoA]. Following, PERMANOVA was conducted with 999 permutations to determine significance of Bray-Curtis PCoA using 'vegan' in R (Oksanen et al., 2007). Differential abundances between treatments for bacterial, archaeal, and protozoal communities individually were calculated using R package 'DESeq2' (Anders and Huber, 2010, Love et al., 2014). This package takes data from taxa tables and raw count information to perform an internal normalization of communities. This is done by calculating a geometric mean across all samples, and then the counts for each gene in each sample are divided by the mean. The size factor of samples can be calculated by looking at the median of the ratios in a sample. Importantly, DESeq2 works to correct RNA composition bias as well as library size in order to target small genes that may be expressed in one sample but not others.

Further, DESeq2 utilizes shrinkage estimation for dispersions and fold changes to account for replicates. A negative binomial generalized linear model is fit for each gene and then the Wald test is used for significance testing. Outliers are automatically removed using Cook's distance and genes that do not meet the threshold of normalized counts are also removed in order to improve the detection power present in DESeq2. Thus, DESeq2 was used to calculate differential abundance in microbial communities.

To visualize raw UHPLC-HRMS data and identify rumen metabolites, an open source metabolomics software package with a grouping algorithm and peak alignment feature, Metabolomic Analysis and Visualization Engine (MAVEN), was used. Metabolites were identified in MAVEN based on the exact mass and retention time of each metabolite (Clasquin et al., 2012). For all 115 identified metabolites, area under the curve was integrated and these data were used for further analysis. Data from MAVEN were processed and analyzed in Metaboanalyst 4.0 (Chong et al., 2018), with significant pathways identified using a global test with relative-betweenness centrality topology analysis with the reference library of *Escherichia coli* K-12 MG1655 (Kanehisa et al., 2013). Further,

metabolites were analyzed for similarity using a principal components analysis [PCA]. Rumen metabolites were imported into SAS v9.4 (SAS Institute, Cary, NC, USA) and normality was assessed using the PROC UNIVARIATE procedure on raw, untransformed data. Normally distributed data were identified using Shapiro-Wilks [W] score of ≥ 0.85 as well as visual analysis of histograms and residuals. Non-normal data ($W < 0.85$) was then log-transformed and analyzed with a mixed model analysis of variance [ANOVA] to compare between moderate and aggressive implant strategies with a covariate of start weight. To address multiple testing, a Fisher's-protected LSD was used with a $P < 0.05$. Significance was determined using $P = 0.05$.

To identify significant metabolites between the moderate and aggressive implant treatment in metabolites with low abundances, information was imported in SAS v9.4 (SAS Institute, Cary, NC, USA) and converted to binary to determine presence or absence of low-abundance metabolites. PROC UNIVARIATE procedure was used to visually analyze histograms, followed by PROC FREQ to analyze abundances of metabolites in each treatment. Samples with low levels of abundance were removed. When low-abundance metabolites were found important between moderate and aggressive implant strategies, least means separation was used to determine significance between moderate and aggressive treatments. Significance was determined using $P = 0.05$. Then, ANOVA was used in SAS to test differences in ADG between treatments of a moderate or aggressive implant strategy. Least means separation was then used to differentiate between the ADG of moderate and aggressive implant strategies. Significance was determined using $P = 0.05$.

Results

Steer Performance

Steers implanted with the aggressive implant strategy had significantly greater ADG than steers implanted with the moderate implant strategy ($P < 0.02$). The ADG for the aggressive implant strategy was 1.72 ± 0.02 kg and the ADG for the moderate implant strategy was 1.66 ± 0.02 kg.

Bacterial Communities

After filtering and processing in R, there were 462 sequence variants identified from 50,838 unique sequences for 336 samples. Chimeras were identified to represent 10% of those sequences. Taxonomy was assigned from the SILVA v132 16S database. After taxonomic assignment, 20,149 taxa were identified within 6 taxonomic ranks. Following taxonomic assignment, alpha- and beta-diversity metrics were measured to indicate richness and diversity. Observed richness of

bacteria was determined from samples, estimated richness of bacterial communities was calculated using Chao1, and samples were analyzed for bacterial richness and diversity using Shannon diversity indices (Figure 2.1). Richness (Shannon) was different between moderate and aggressive implant strategies ($P < 0.01$), however Chao1 and observed species were not ($P > 0.05$). Following, a principal coordinates analysis (PCoA) identified any dissimilarity among clustered samples, using Bray-Curtis distance method (Figure 2.2). Beta-diversity was analyzed with PERMANOVA for Bray-Curtis PCoA with no similarity between treatments ($P > 0.05$).

There were no significant differences between the moderate and aggressive implant strategies amongst any taxonomic level after analyses through DESeq2. The majority of bacterial phyla identified in the rumen fluid were Bacteroidetes and Firmicutes (59-60% and 27-28%, respectively) as shown in Figure 2.3. The Proteobacteria accounted for 11-12% of reads, with less abundant phyla identified as Spirochaetes, Fibrobacteres, and Actinobacteria. Many phyla represented less than 1.0% of relative abundance. Relative abundances represented in Figure 2.3 represent abundances out of the top 10 phyla present in bacterial rumen samples.

Identified bacterial genera (Figure 2.4) consisted primarily of *Prevotella*, making up 44-45% of samples. This was followed by *Succinivibrionaceae* (10-12%), and then taxa ranking less than 1% of abundances, being those related to the family *Lachnospiraceae*, *Shuttleworthia*, those related to the family *Ruminococcaceae*, and *Oribacterium*. Presence of other genera were less than 1% abundance. Unassigned taxa at the genus level made up 0.4% of total taxa. Visual analysis of Figure 2.4 in addition to performing differential abundance analyses supports the lack of change in the bacterial communities between moderate and aggressive implant strategies.

Archaeal Communities

For archaeal communities, after filtering and processing, there were 56 sequence variants inferred from 7,839 unique sequences for 336 samples. Taxa were assigned using SILVA v132 for 16S data. After taxonomic assignment, 642 taxa were identified at 6 taxonomic ranks. Alpha- and beta-diversity metrics were performed on archaeal communities. Alpha-diversity metrics of observed archaeal abundances, Chao1 (expected abundances) and Shannon (richness and diversity) were calculated and not statistically different between treatments ($P > 0.05$) (Figure 2.5). Beta-diversity was measured through a Bray-Curtis PCoA, visually demonstrating the similarity between treatments (Figure 2.6). The PERMANOVA performed on Bray-Curtis PCoA showed no significance between differences in moderate and aggressive implant strategies ($P > 0.05$).

There were no significant differences between the moderate and aggressive implant strategies at any taxonomic level after performing analyses in DESeq2. The majority of archaea at the phylum level were Euryarchaeota, making up 99% of samples. At the genus level, the majority consisted of *Methanobrevibacter* (93-95%), with smaller abundances of *Methanosphaera*, *Candidatus Methanomethylophilus*, and those related to the family *Methanomethylophilaceae* (Figure 2.7). Unassigned reads made up 0.3% of reads at the genus level.

Protozoal Communities

After filtering and processing in R for protozoal communities, there were 14 sequence variants inferred from 10,575 unique sequences for 208 samples. As previously mentioned, low-quality samples that did not meet filtering criteria were removed. Taxa were assigned using SILVA v132 for 18S data. After taxonomic assignment, 220 taxa were identified at 6 taxonomic levels. Alpha-diversity was measured for observed protozoal abundances, Chao1 (expected abundances) and Shannon (richness and diversity) (Figure 2.8). Alpha-diversity between moderate and aggressive implant strategy was not found to be statistically significant ($P > 0.05$). Beta-diversity was measured using a Bray-Curtis PCoA for distance (Figure 2.9), demonstrating similarity between samples. Dissimilarity between moderate and aggressive implant strategies was insignificant after PERMANOVA was performed on beta-diversity ($P > 0.05$).

At the phylum level, SAR was the most abundant category (99%). At the genus level, there were significant differences ($P < 0.01$) between *Isotricha* in the moderate and aggressive implant strategy. The *Isotricha* made up $2-3\% \pm 2.7-2.9\%$ of genera in protozoal communities. Greater abundances of the *Isotricha* were noted in the moderate implant strategy. Other genera were not significantly different between implant strategies, but consisted of *Entodinium* (58-60%) *Opyrsclex* (36-40%), and *Polyplastron* (>1%). Unassigned taxa at the genus level consisted of 0.02% of total taxa.

Rumen Metabolite Data

After untargeted metabolomics were performed on rumen samples, 115 unique metabolites were found. Visual analysis of the partial least squares determinant analysis [PLS-DA] showed little separation between moderate and aggressive implant strategies (Figure 2.11). Principle component analysis of the 115 metabolites demonstrated similarity in the metabolic profile of the moderate and aggressive implant strategies (Figure 2.12). For the moderate and aggressive implant strategies, two metabolites were found significant in the moderate implant strategy (Table 2.3). In the moderate implant strategy treatment group, acetyllysine and N-acetylornithine were found significantly greater ($P \leq 0.03$ and $P \leq 0.04$,

respectively). The covariate of starting weight was not significant in the model ($P > 0.05$).

Table 2.3. Metabolites differing between aggressive and moderate implant strategies

Metabolite	Aggressive Treatment^b	Moderate Treatment^b	<i>P</i>-value^a
Acetyllysine	78,453±5606.94	98,259±5606.94	0.0291
N-Acetylornithine	96,563±5006.59	110,899±5006.59	0.0364

^a Significance determined at $P \leq 0.05$

^b Values measured as mean \pm SEM of the area under the peak

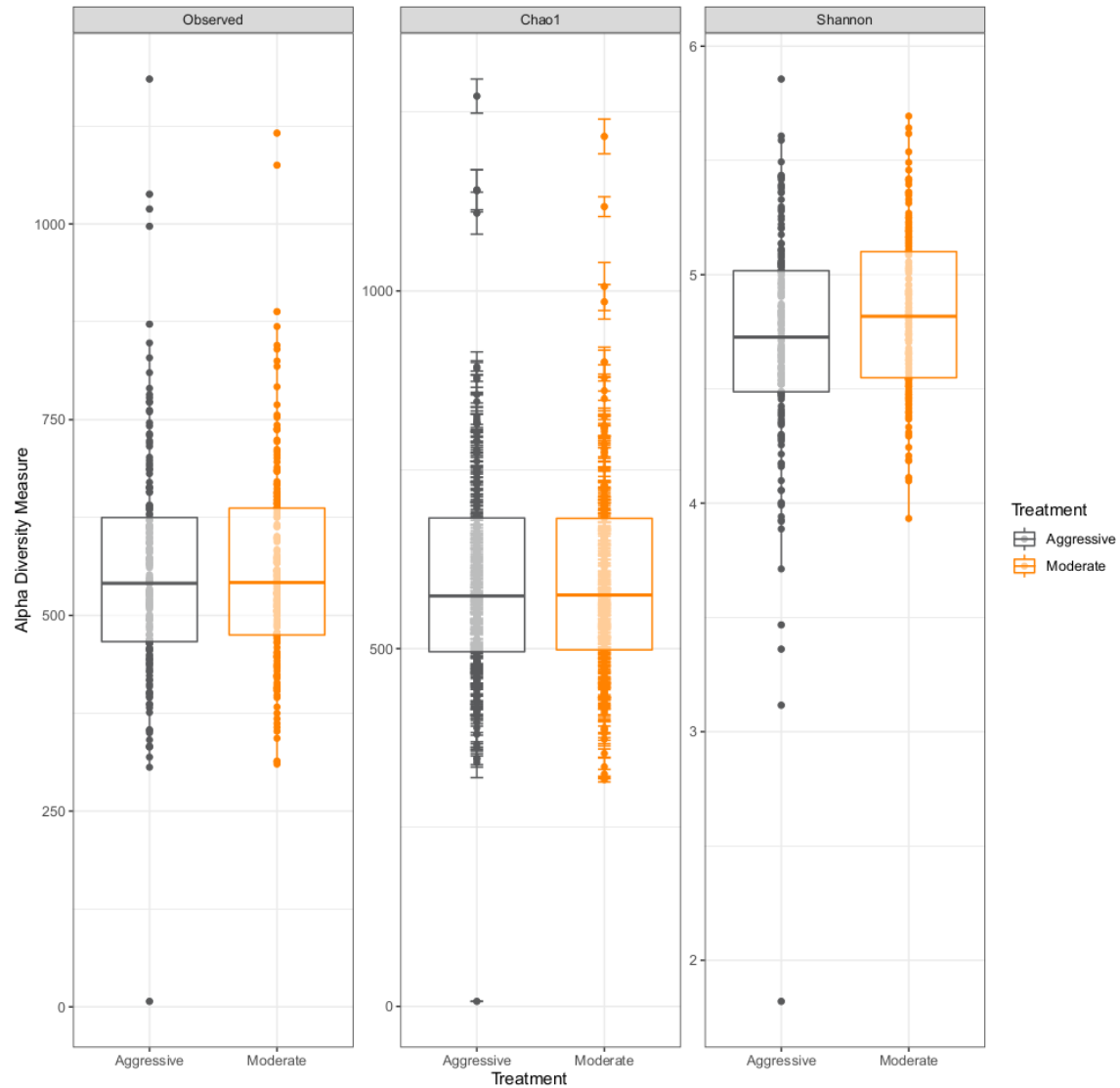


Figure 2.1. Alpha-diversity metrics for bacterial communities

Alpha-diversity metrics for bacterial communities with observed abundances of communities, expected abundances (Chao1), and richness and diversity measured with Shannon diversity index. Aggressive treatments are represented in grey and moderate treatments are represented in orange. Significant differences were only noted in the Shannon diversity index between treatments ($P < 0.05$).

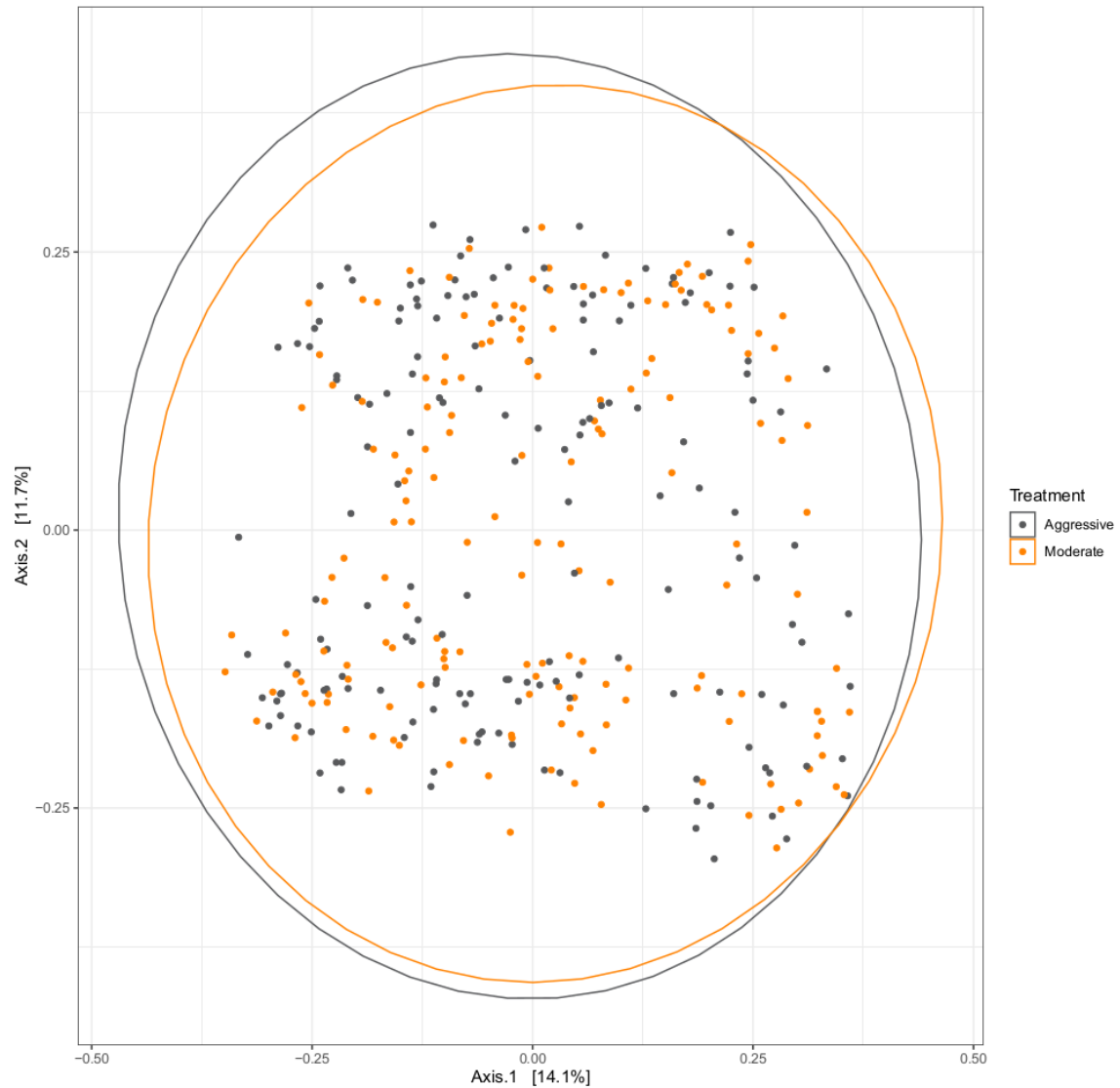


Figure 2.2. Beta-diversity measurements for bacterial communities

Beta-diversity measurements for bacterial communities using Bray-Curtis distances to create a PCoA. Aggressive treatments are represented in grey and moderate treatments are represented in orange. Ellipses represent 95% confidence intervals around means for aggressive and moderate implant strategies.

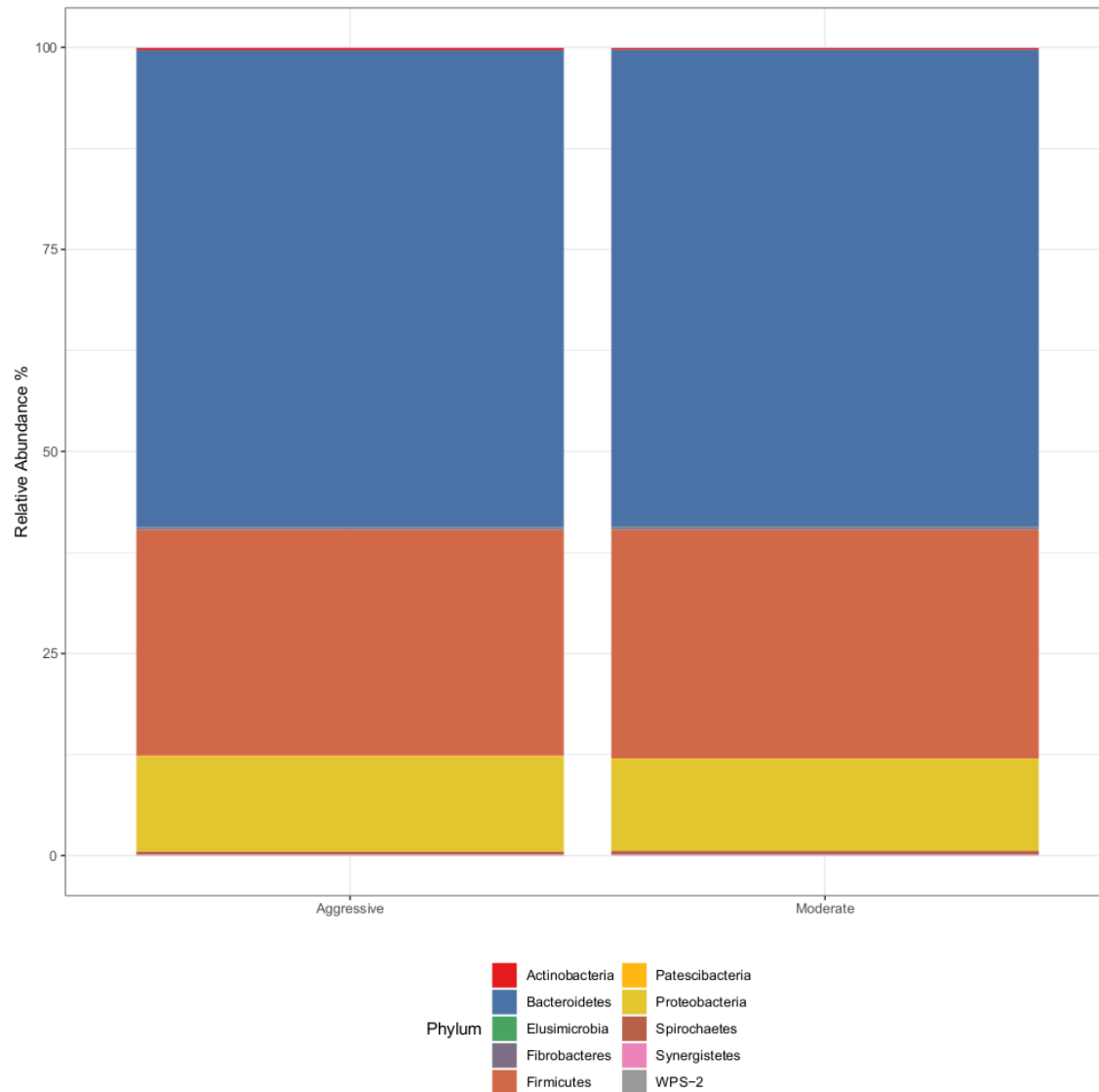


Figure 2.3. Average relative abundance of phylum-level bacterial communities identified in ruminal fluid

Average relative abundance of the top ten phylum-level bacterial communities classified in ruminal fluid between aggressive or moderate implant strategies. Relative abundance was calculated by reads of taxon/total reads in a sample.

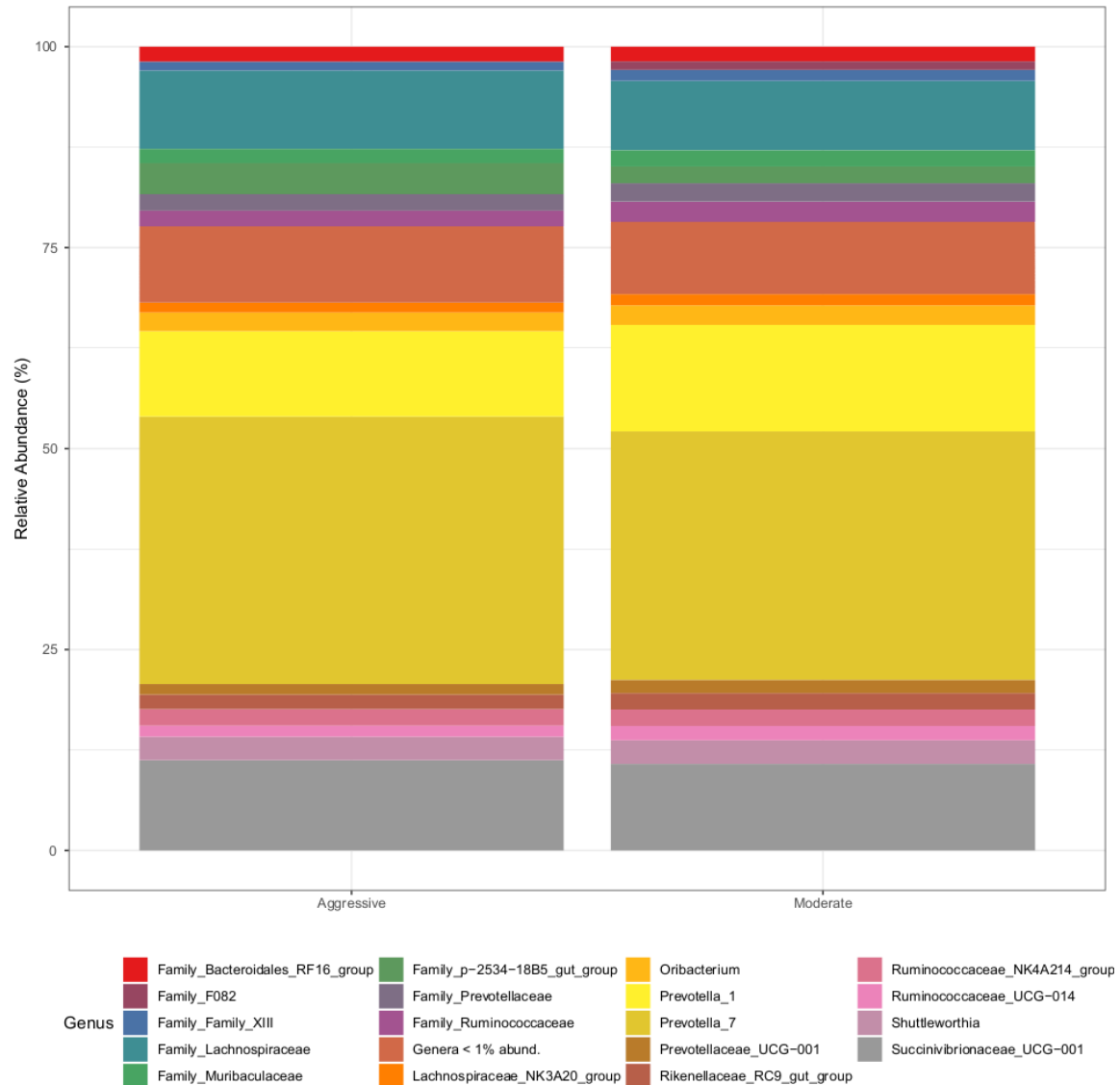


Figure 2.4 Average relative abundance of genus-level bacterial communities identified in ruminal fluid

Average relative abundance of the genus-level bacterial communities classified in ruminal fluid between aggressive or moderate implant strategies. Relative abundance was calculated by reads of taxon/total reads in a sample to compare between treatments. Genera accounting for less than 1% abundance were grouped.

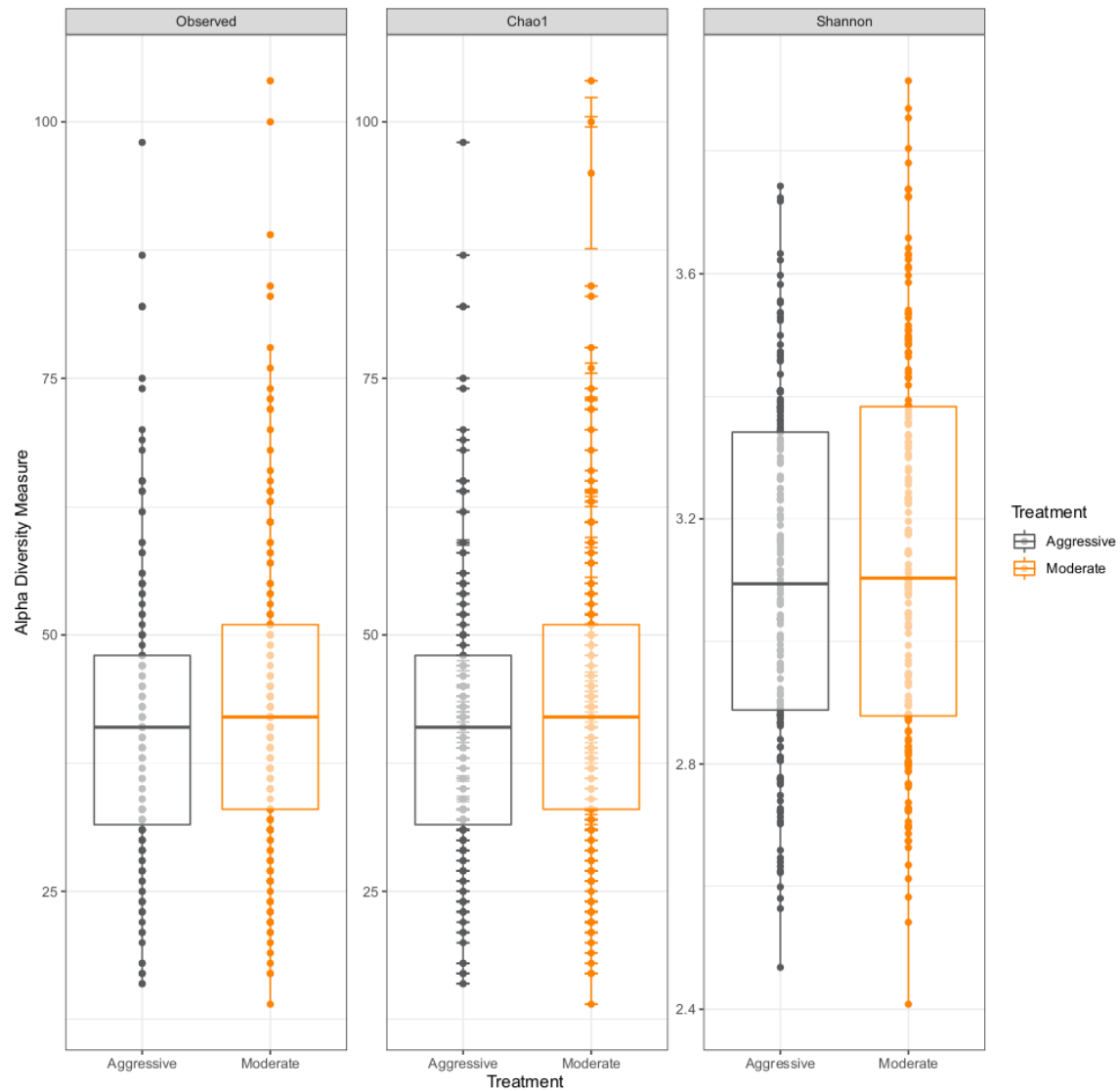


Figure 2.5. Alpha-diversity metrics for archaeal communities

Alpha-diversity metrics for archaeal communities with observed abundances of communities, expected abundances (Chao1), and richness and diversity measured with Shannon diversity index. There were no significant differences between alpha-diversity metrics between moderate and aggressive implant strategies. Aggressive treatments are represented in grey and moderate treatments are represented in orange.

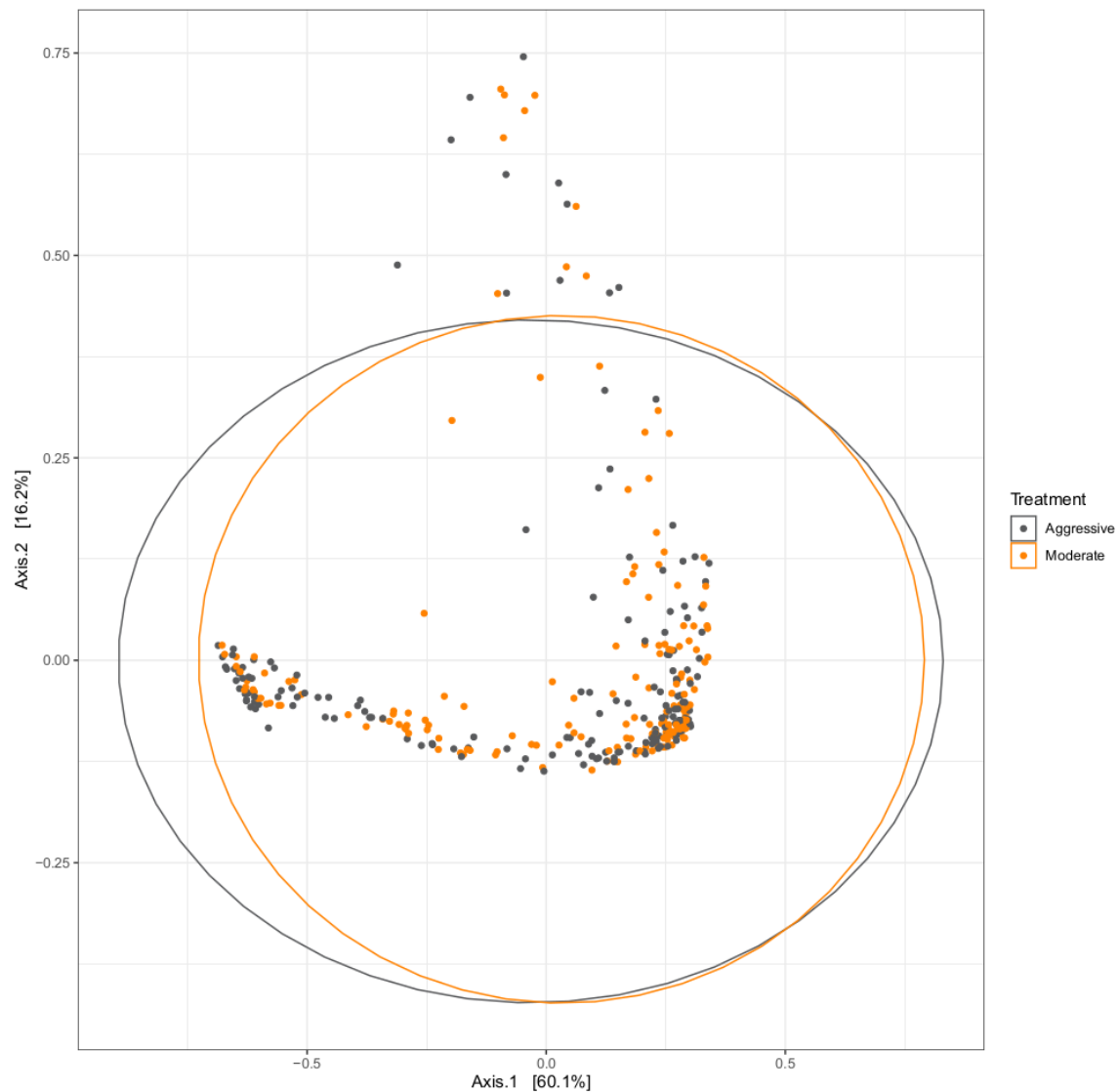


Figure 2.6. Beta-diversity measurements for archaeal communities

Beta-diversity measurements for archaeal communities using Bray-Curtis distances to create a PCoA. Aggressive treatments are represented in grey and moderate treatments are represented in orange. Ellipses represent 95% confidence intervals around means for aggressive and moderate implant strategies. Significant overlap between communities reveals no differences between moderate or aggressive treatments.

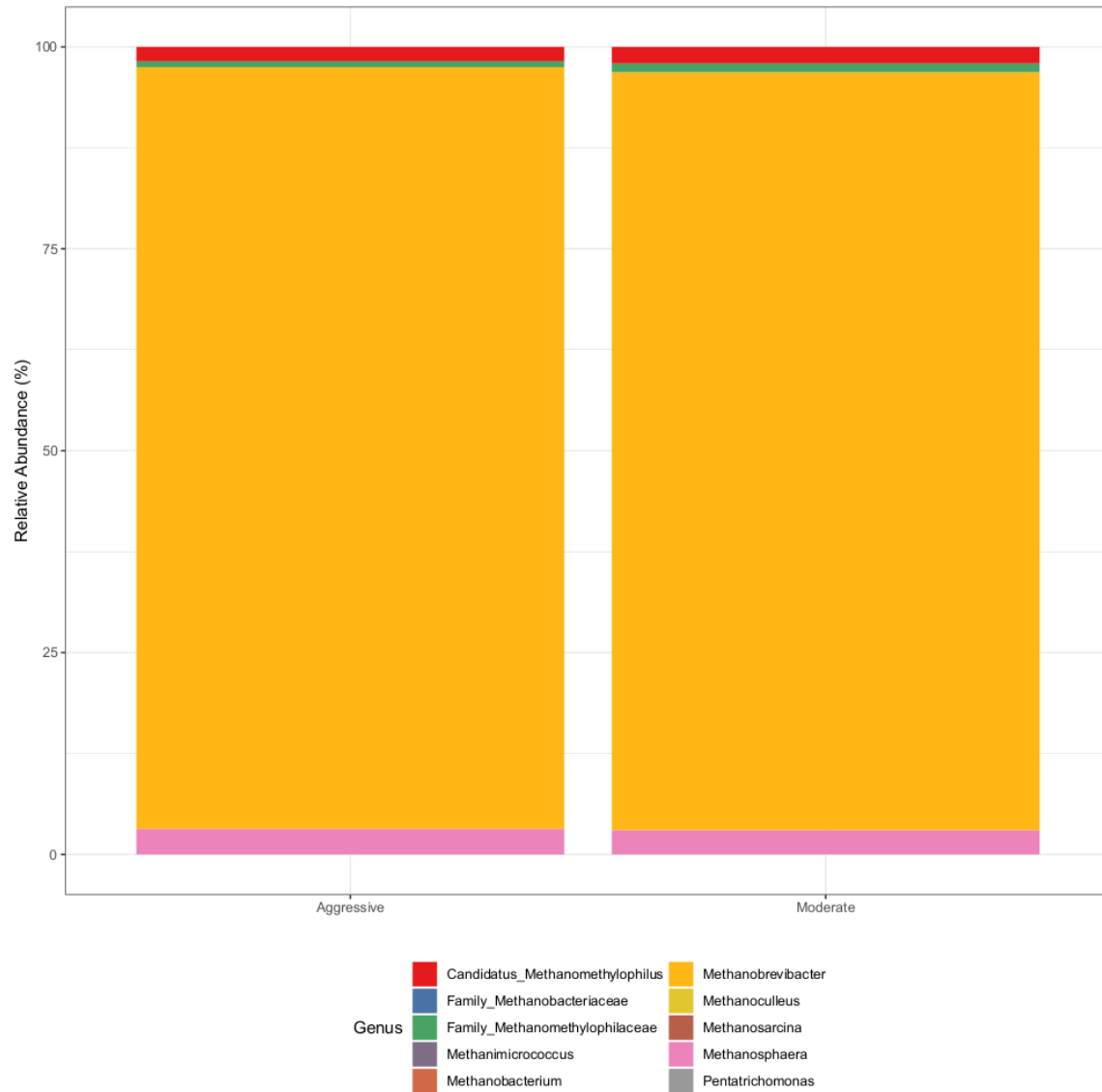


Figure 2.7. Average relative abundance of the genus-level archaeal communities identified in ruminal fluid

Average relative abundance of the genus-level archaeal communities classified in ruminal fluid between aggressive and moderate implant strategies. Relative abundance was calculated by reads of taxon/total reads in a sample to compare between treatments.

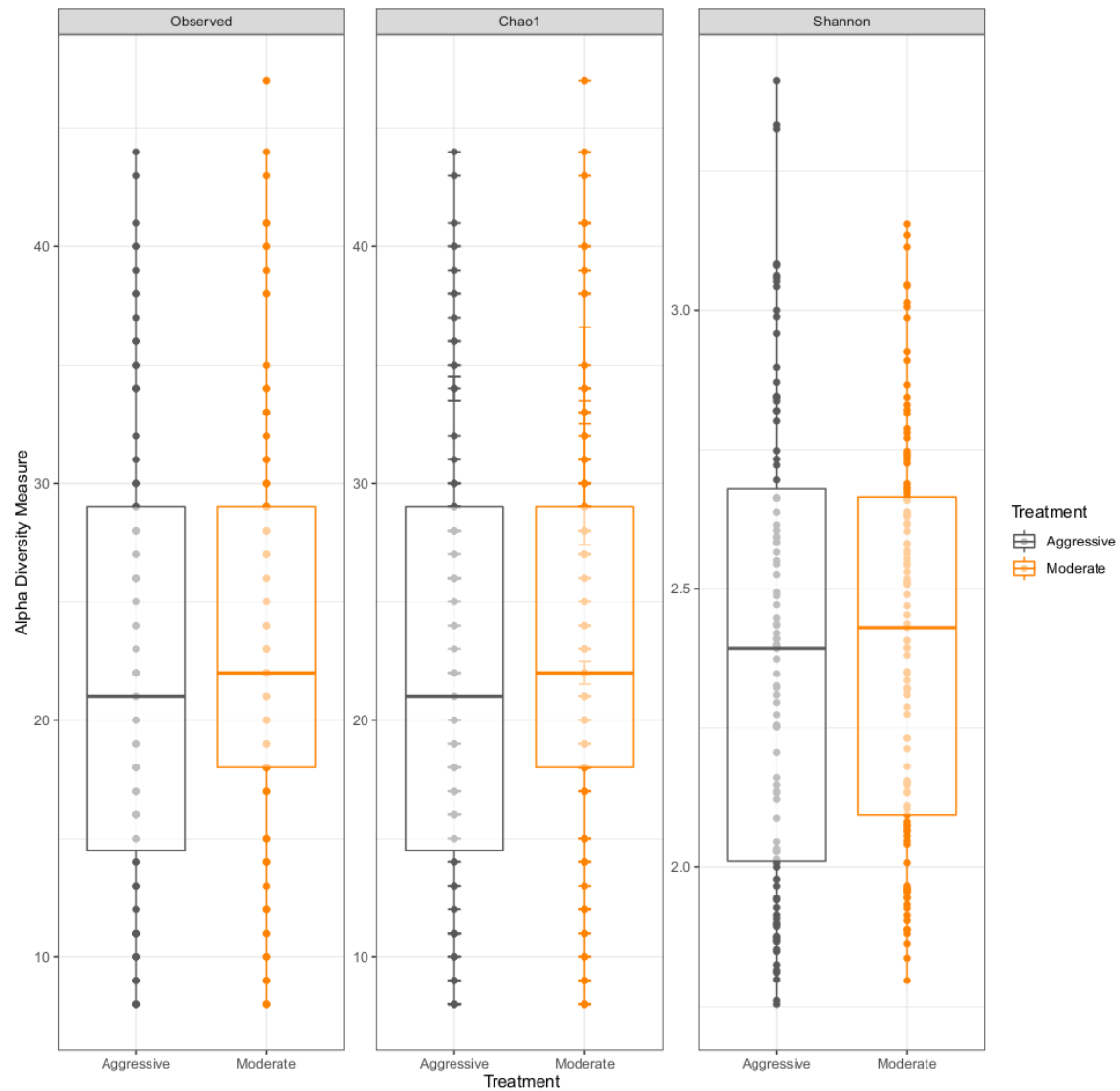


Figure 2.8. Alpha-diversity metrics for protozoal communities

Alpha-diversity metrics for protozoal communities with observed abundances of communities, expected abundances (Chao1), and richness and diversity measured with Shannon diversity index. There were no significant differences between alpha-diversity metrics between moderate and aggressive implant strategies. Aggressive treatments are represented in grey and moderate treatments are represented in orange.

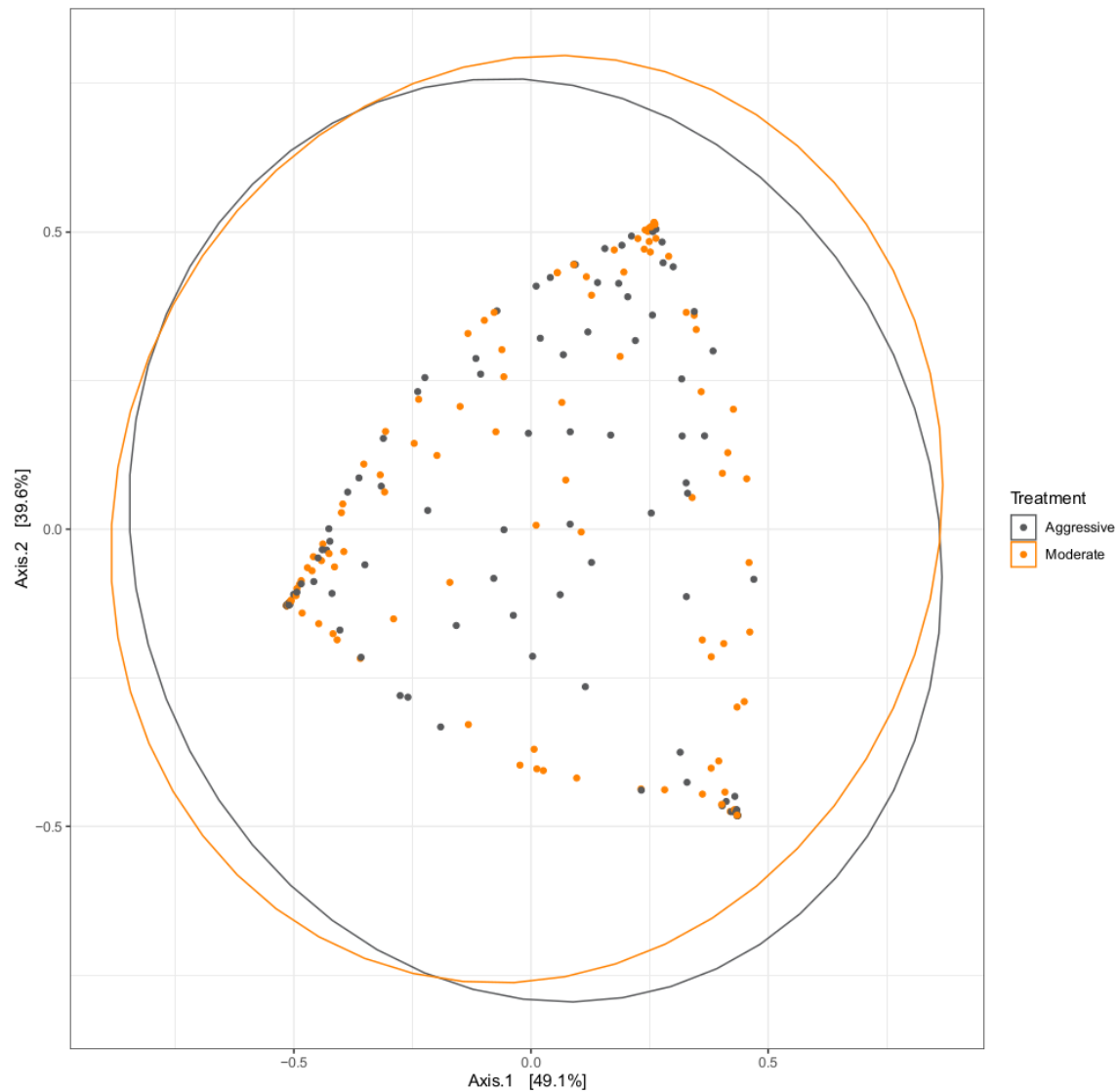


Figure 2.9. Beta-diversity measurements for protozoal communities

Beta-diversity measurements for protozoal communities using Bray-Curtis distances to create a PCoA. Aggressive treatments are represented in grey and moderate treatments are represented in orange. Ellipses represent 95% confidence intervals around means for aggressive and moderate implant strategies. Significant overlap between communities reveals no differences between moderate or aggressive treatments.

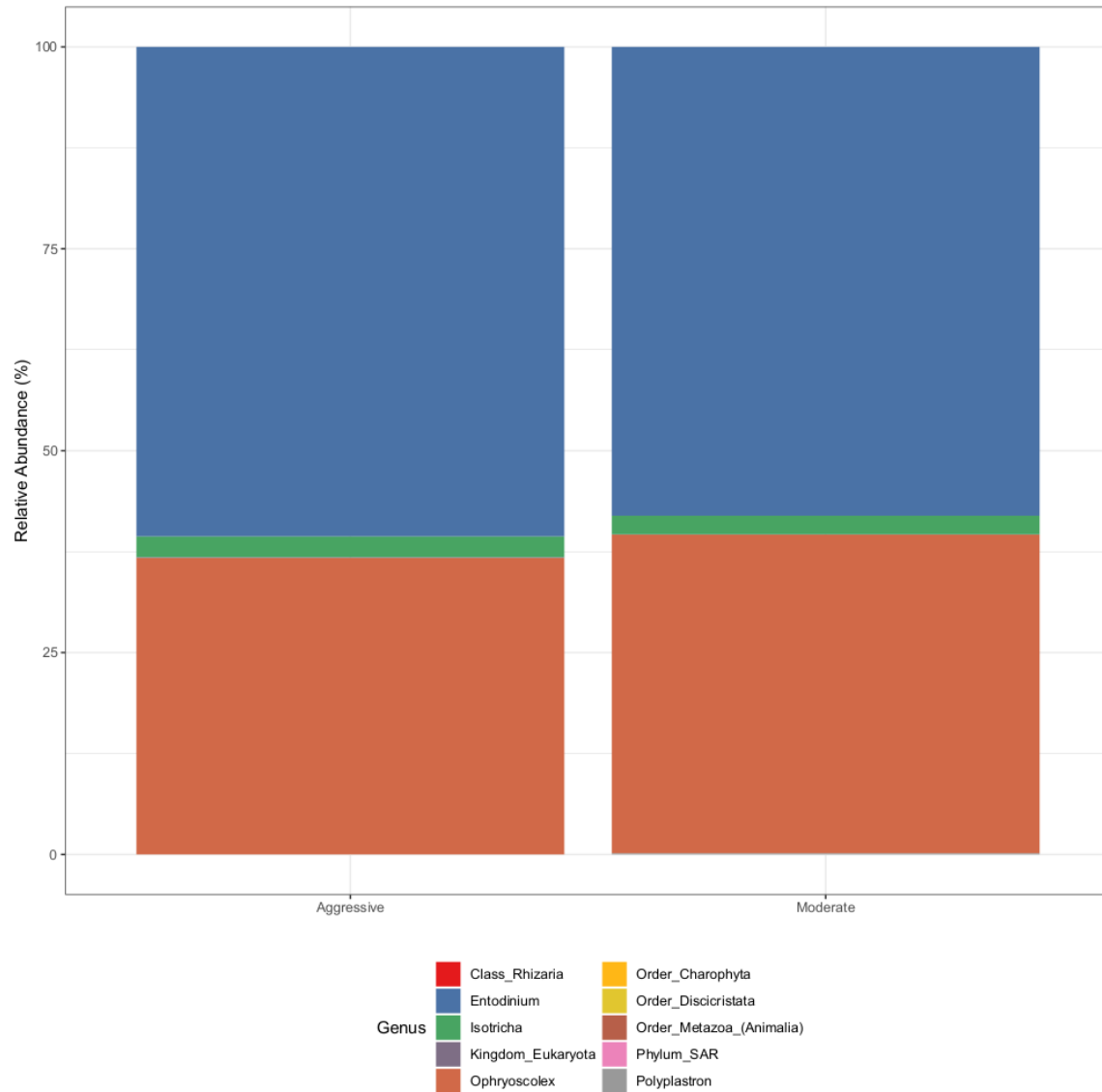


Figure 2.10. Average relative abundance of genus-level protozoal communities identified in ruminal fluid between aggressive or moderate implant strategies

Average relative abundance of the genus-level protozoal communities classified in ruminal fluid between aggressive and moderate implant strategies. Relative abundance was calculated by reads of taxon/total reads in a sample to compare between treatments.

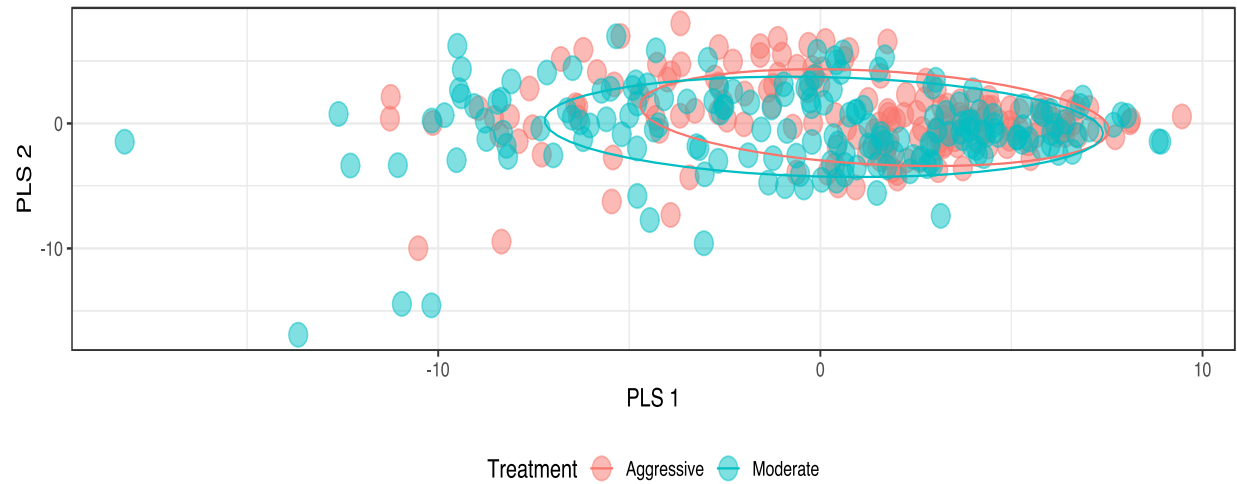


Figure 2.11. Partial least squares discriminant analysis of rumen metabolites

Partial least squares discriminant analysis [PLS-DA] of rumen fluid metabolites illustrating overlap between the moderate and aggressive implant strategies. Ellipses represent a 95% confidence interval around mean of the data. Aggressive strategies are represented in red and moderate strategies are represented in blue.

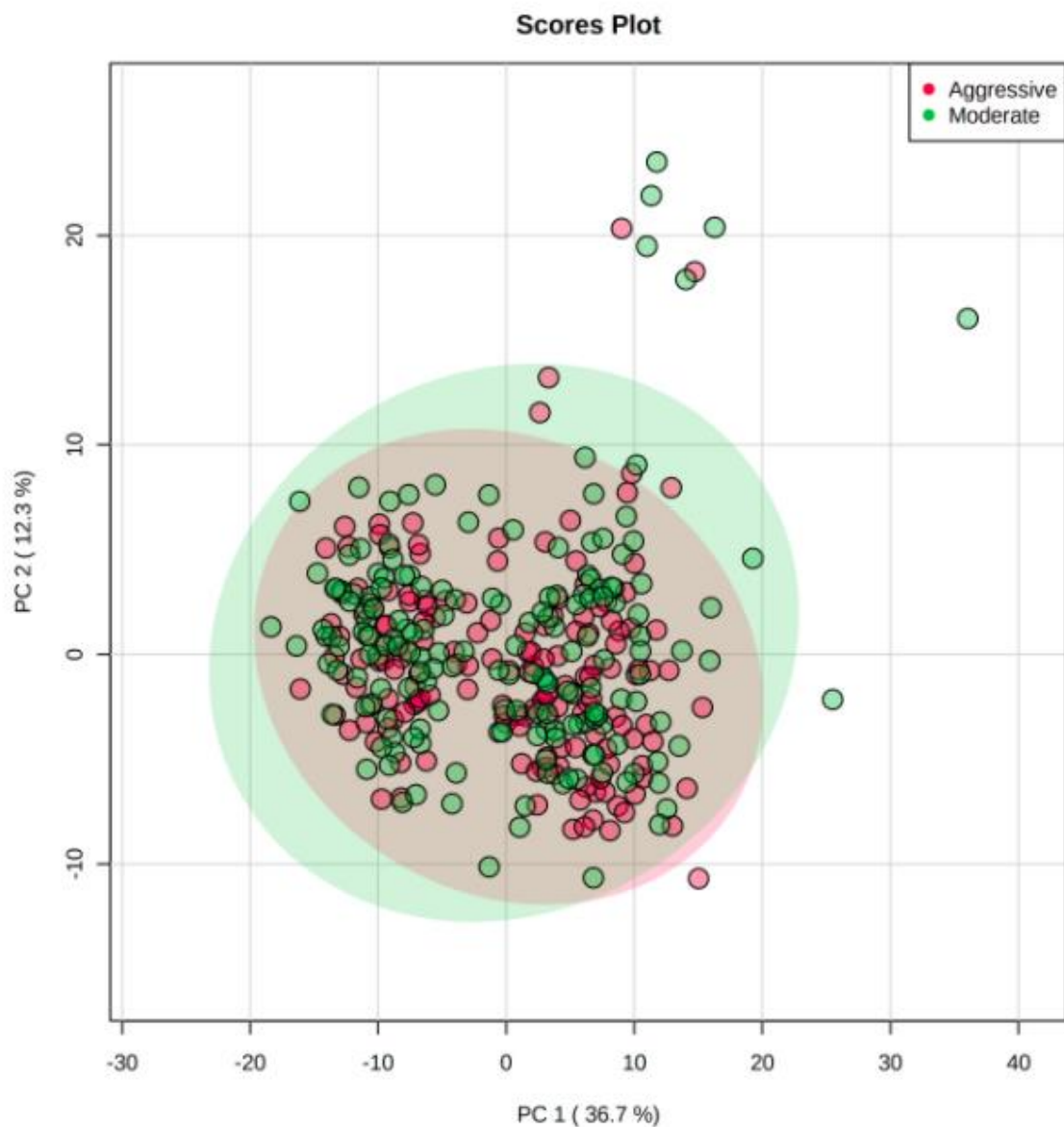


Figure 2.12. Principal component analysis of rumen metabolites

Principal component analysis [PCA] of 115 rumen fluid metabolites identified through untargeted metabolomics. The aggressive implant strategy is represented in red and the moderate implant strategy is represented in green. Significant overlap of metabolites present in moderate and aggressive implant strategies illustrates similarity of rumen fluid metabolic profiles. Shaded regions represent a 95% confidence interval around the metabolites per treatment strategy.

Discussion

Improving the efficiency of weight gain and growth in beef cattle could offer a solution for the need to provide a reliable protein source to feed a growing population. Growth-promoting implant strategies remain one of the most effective ways to improve gain in beef cattle, increasing ADG up to 30% and feed efficiency by 15% (Preston, 1999). The use of aggressive implant strategies is thought to improve ADG beyond the capabilities of moderate implant strategies due to increased ratios of androgenic and estrogenic compounds. Aggressive implant strategies have been previously critiqued for their effect on carcass composition, specifically in reduction to marbling scores and meat tenderness (Jones et al., 1991, Dikeman, 2007). Selecting for a moderate or aggressive implant strategy could affect an operation through cost of implant and overall live carcass weights and quality. Thus, this study allowed in part the use of ADG between moderate and aggressive implant strategies to understand potential growth differences in strategies.

Although ADG was statistically different between the two treatment groups, for every 1 kg gained by the moderate implant strategy group, 1.03 kg was gained for the aggressive group. This minor difference between the treatments would likely not influence a producer's decision to select either the moderate or aggressive strategy for ADG. An aggressive implant strategy requires that animals stay in the operation longer and thus are associated with high feed costs that could be detrimental to a producer. Previous reviews have discussed the necessity of understanding the costs and benefits for aggressive implant strategies, as they are generally not as well-suited to all beef cattle operations (Reinhardt, 2007).

The use of growth-promoting hormones in management strategies may be influencing other factors of weight gain and feed efficiency in beef cattle, such as the rumen microbiome. The rumen microbiome has been previously identified to alter feed efficiency in beef cattle through the conversion of nutrients to energy for the host (Mizrahi, 2011, Myer et al., 2015). Further, fermentative products of the rumen microbial communities act to provide energy to the host as well as play a role in metabolic pathways. Microbial communities did not differ between the moderate and aggressive implant strategy for both bacterial and archaeal communities. No significant differences in bacterial communities were noted when looking at beta-diversity for the 336 samples. Notably, while alpha-diversity for bacterial communities did differ, this is likely not biologically relevant as no microbial communities differed between treatments. This is demonstrated by the alpha-diversity between samples, and beta-diversity showing a large overlap between the clustered sequences.

The lack of differences in the rumen microbiome and metabolome may be explained by the diet and similarity of implant strategies given to cattle. While these

implant strategies are identified as moderate and aggressive, there was overall little differences in ADG to explain the significance in these values. The aggressive Revalor implant strategies have been previously thought to be more effective in beef cattle remaining in feedlot operations for longer periods of time to see an improved payoff (Hilscher et al., 2016). Importantly, this extended time spent in feedlot allows for coated aggressive implants to reach their full capacity, altering the ADG in these cattle. With this in mind, animals receiving the same diet and these implant strategies may result in little difference in microbial and metabolic profiles. Previous studies conducted have demonstrated a similar rumen microbiome composition across animals in different climates, farming practices, and diets (Henderson et al., 2015). In studies with animals receiving the same diet and no other treatment, rumen microbiome profiles would be expected to be very similar. However, diet would not be the only contributing factor in a lack of differences in microbial communities present in the rumen.

Previous research has not identified estrogenic and androgenic receptors on the rumen epithelium of mature cattle, potentially meaning that there is not a mechanism for these hormones to enter the rumen environment. While estrogen-like receptor- α , highly similar in sequence similarity to estrogen receptors (Giguère et al., 1988), has been researched on the rumen epithelial in calves aging from 14-42 days of age, (Connor et al., 2014), these studies have not identified other binding sites for estrogenic and androgenic compounds found in growth-promoting implants. Further, the estrogen-related receptors have not been identified to bind to estrogens or related steroid hormones, but function to increase transcription of genes in energy metabolism through an unknown endogenous ligand (Schreiber et al., 2003). There is a need for studies to report on receptors present on rumen tissue functioning to allow interaction between growth-promoting hormones and the rumen environment.

The presence of estrogenic receptors on gastrointestinal tissues has been identified in human studies, prompting further research into the gut-brain axis involving activity of estrogens. Decreases in the diversity in the gut microbiome in humans have been linked to a reduction of β -glucuronidase, which functions to deconjugate estrogen and phytoestrogens into their active and circulating forms (Baker et al., 2017). If these estrogenic receptors can be identified on rumen tissue, there is potential for additional circulating estrogen to spur additional GH and IGF-1 to increase muscle tissue growth through binding sites present on muscle tissue. Potentially, the interaction of between the microbial communities and estrogenic compounds in humans could lead to studies determining the presence of these receptors in ruminants.

In bovines, androgenic compounds have been demonstrated to have direct and indirect effects on muscle and growth response through binding directly to receptors in muscle tissues (Sauerwein and Meyer, 1989), while indirectly spurring

production of GH and IGF-I (Grigsby and Trenkle, 1986). Bovine skeletal muscle directly interacts with free androgens that are introduced via implanting strategies through androgen receptors, which in addition to estrogen, can cause additive growth in muscle tissue (Snochowski et al., 1981). While GH and IGF-I play an important role in muscle development, they have not been associated with rumen functionality. Research is still needed to identify potential receptors in the rumen in order to draw conclusions.

While the protozoal genera *Isotricha* was found in greater presence in the moderate implant group, no other microbial community was found significantly different between treatments. Interestingly, past studies have looked at the defaunation of protozoal communities to understand their impact on the rumen microbiome (Newbold et al., 2015). The protozoal communities present in the rumen often outweigh their positive interaction with digestion of fiber and starch particles (Mendoza et al., 1993, Takenaka et al., 2004) by decreasing efficient N utilization in the rumen, thus decreasing energy provided to the host. Further, ruminal protozoa function to phagocytize some bacterial communities, which decreases the microbial protein (Ushida et al., 1990). Despite interesting functionality behind host energy supply and protein metabolism from protozoal communities, the *Isotricha* populations cannot be directly associated with differences in the metabolome or gain and influences can only be speculated.

Out of the 115 metabolites identified in rumen fluid, only two were significantly different. While there were significant differences in metabolites, it is important to note that there were no significant differences between bacterial and archaeal communities in the rumen microbiome. Thus, the differences in metabolites likely does not stem from rumen bacterial or archaeal activity. With only one protozoal genus differing between the moderate and aggressive implant strategy, significant metabolites are more likely due to host metabolic activity rather than any microbial activity or fermentation. Acetyllysine is an acetyl-derivative of lysine, an essential amino acid that is crucial for growth in beef cattle. Interestingly, lysine functions as one of the most limiting amino acids in growing cattle (Richardson and Hatfield, 1978), as it contributes greatly to maximizing lean tissue deposition (Friesen et al., 1994). The acetylation of lysine may explain why the moderate implant treatment resulted in lower ADG, as less lysine may be contributing towards muscle growth. However, this is likely not the sole explanation for the decrease in ADG in the moderate implant treatment group, as amounts of androgenic and estrogenic hormones directly influence skeletal muscle by stimulating GH and IGF-I to bind to muscle, causing proliferation of muscle cells and resulting in additional growth (Froesch and Zapf, 1985). Thus, the use of growth-promoting implant strategies in beef cattle is more likely to influence ADG than the presence of metabolites in rumen fluid.

Previous studies analyzing how shifts in protozoal communities may influence rumen metabolites have commonly found purine derivatives, due to their influence on microbial protein synthesis (Chen and Gomes, 1992). Interestingly, the metabolites identified in this study that could be due to significant shifts in protozoal communities were not identified in other studies. Purine derivatives, such as allantoic acid and hypoxanthine, were identified (Chen and Gomes, 1992), however did not significantly differ between treatments. In addition, metabolites that have been previously identified to be influenced by protozoa in lambs such as aminoadipate, cholate, and pantothenate did not significantly differ in this study (Morgavi et al., 2015). Importantly, aminoadipic acid has been identified to be influenced by protozoal communities, and plays an important role in lysine metabolism. While this metabolite was not significantly different between treatments, acetyllysine also affects presence of lysine in biological fluids.

While previous studies have analyzed rumen metabolites (Artegoitia et al., 2017, Clemmons et al., 2020), the pathways and activity of many of these metabolites play multiple functions throughout the rumen and host metabolism, and thus can be difficult to identify true functionality. In addition, other studies have included that the origin of the metabolites can be difficult to trace, with half of the rumen metabolites coming from bovine origin and the other half from microbial origin (Saleem et al., 2013). Further, previous studies have also found wide variation between the abundance of metabolites, leading to believe that much more research will be required to be able to associate rumen metabolites with diet and feed efficiency (Saleem et al., 2013). While the presence of acetyllysine may be due to shifts in protozoal communities, other metabolites such as N-acetyloronithine could be due to differential absorption across the rumen epithelium and may be of host origin due to a lack in differences in bacterial and archaeal communities between the moderate and aggressive implant strategies as well as the small differences in ADG between the steers. Notably, there are few studies involving N-acetyloronithine and its metabolic effect on beef cattle, making associations more difficult. Previous studies have suggested the relationship of N-acetyloronithine with enzymatic activity for arginine and lysine biosynthesis (Ledwidge and Blanchard, 1999), which may be contributing towards protein use in cattle. However, this information is not readily available in ruminants, and thus is only a potential association.

Understanding the relationship of microbial communities and host phenotype for feed efficiency and growth may allow for better identification of efficient beef cattle. While this study serves to identify differences in microbial communities between moderate and aggressive implant strategies, crucial information regarding implant effects on the rumen is lacking due to focus in physiological responses via muscle tissue and hormones such as GH and IGF-I. Importantly, nutritional physiology plays an important role in determining and influencing gain in beef cattle, and thus

identifying this relationship is critical to understanding implant effects on all physiological systems.

Conclusions

Previous studies have identified that the use of growth-promoting implants greatly improves ADG in beef cattle, making it one of the more effective management strategies in increasing growth. Additionally, studies have identified the importance of the rumen microbiome in nutrient conversion and overall feed efficiency. Between a moderate and aggressive implant strategy, only one protozoal genus differed, potentially indicating that the amounts of growth-promoting hormones from implant strategies do not influence the rumen, likely due to a lack of androgen and estrogen receptors on rumen tissue. With rumen microbial communities producing fermentative products and metabolites that may be linked to phenotypic traits, metabolites should be further characterized in the rumen. While no significant differences were identified between microbial communities, this study identified potential differences in ADG in steers given a moderate or aggressive implant strategy.

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VITA

Madison Henniger was born July 28th, 1997 in Shawnee Mission, KS. Madison graduated high school from Arlington High School in 2015. She attended the University of Tennessee, Knoxville to receive her Bachelor of Science in Animal Science with the 5 Year B.S./M.S. in May 2019. Madison began her Master of Science in Animal Science at the University of Tennessee in Summer 2018. Madison worked with Dr. Phillip Myer for her Master of Science in Animal Science, and graduated with her degree in Summer 2020.